Author:
Arias Robledo, Gerardo

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Lucilia blowflies

their ecology, taxonomy and the evolution of obligate amphibian parasitism

Lucilia blowflies: their ecology, taxonomy and the evolution of obligate amphibian parasitism

Gerardo Arias Robledo

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Abstract

Blowflies (Diptera: Calliphoridae) are of evolutionary, ecological and economic importance, performing essential ecosystem services in the consumption, recycling and dispersion of carrion and acting as facultative agents of livestock myiasis. The interspecific ecological differences that facilitate coexistence within the diverse blowfly community are not fully understood. To quantify differences in habitat use by calliphorid species (Chapter 2), thirty flytraps were distributed within three habitats at two sites in south west England during March–August 2016. A total of 17,246 specimens were caught and identified, *Lucilia sericata* (Meigen) was the dominant species in open habitats, whereas *Lucilia caesar* (Linnaeus) was the most abundant species in shaded habitats. The results demonstrate that *Calliphora* and *Lucilia* species show strong tempo,ral and spatial segregation, mediated by temperature, and that species of the genus *Lucilia* show differences in habitat use which are likely to be driven by differences in humidity tolerance and light intensity. These factors in combination result in effective niche partitioning. Within the genus *Lucilia* only one species is generally recognized as an obligate agent of myiasis in Europe*, Lucilia bufonivora* (Moniez). This species is a specialist parasite of amphibians. However, it has been suggested that a second species *Lucilia silvarum* (Meigen) may also act as a facultative parasite of amphibians. Their morphological similarity has led to misidentification, taxonomic ambiguity and a paucity of studies of *L. bufonivora*. To resolve this question (Chapter 3), larvae were analysed from toad myiasis cases from the U.K., The Netherlands and Switzerland, together with adult specimens of fly species that are thought to be implicated in amphibian parasitism: *L. bufonivora*, *L. silvarum* and the strictly Nearctic *Lucilia elongata* (Shannon). Partial sequences of two genes, *COX1*and *EF1a*, were amplified. Bayesian inference trees of *COX1*and *EF1a* and a combined-gene dataset were constructed. All larvae isolated from toads were identified as *L. bufonivora* and no specimens of *L. silvarum* were found implicated in amphibian myiasis. This study confirms *L. silvarum* and *L. bufonivora* as distinct sister species, however there is not clear resolution on the relationships of *L. silvarum* and *L. elongata* using the nuclear marker *EF1a*. The evolution of obligate toad parasitism is of particular interest and to investigate this (Chapter 5), molecular clock-dating was performed with a concatenated data set of 3 genes: *COX1* (mtDNA), *ITS2* (non-coding) and *per* (nDNA). Unlinked substitution and relaxed clock models were implemented to allow evolution to vary amongst lineages. Obligate amphibian parasitism probably evolved just once around 4 mya. It is likely that this occurred after the niche displacement of a saprophagous ancestor from the carrion-fly community. Evidence from nDNA phylogenies suggest that, although with slow nuclear evolution rates, *L. elongata* is a distinct species to *L. silvarum*. Consistent paraphyly of *L. bufonivora* across single-gene phylogenies and high mtDNA sequence divergence between Palearctic and Nearctic lineages suggest on-going cryptic speciation of *L. bufonivora* in these two regions for at least 2mya. Thus, due to its relative rarity, it has remained unrecorded by taxonomists until recent studies. Since the ecology of *L. bufonivora* is poorly understood, ecological studies were undertaken in the Netherlands (Chapter 5). These demonstrated the low abundance of adult *L. bufonivora* in the field and showed that it was more frequently encountered in open and wet habitats where its hosts are abundant rather than in woodland habitats. The broad issues surrounding the evolution of diversity within the calliphorid blowflies are discussed (Chapter 6) and it is suggested that it is the patchy and ephemeral nature of carrion that is the key to understanding the ecology and evolution of this family of flies, since this drives the evolution of niche partitioning and specialisation.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED……Gerardo Arias Robledo…….....

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1. Introduction: the ecology and taxonomy of Calliphoridae

1.1 Calliphoridae: Blowflies

Calliphoridae, also known as blowflies, are a family of insects in the order Diptera, with over 1000 known species across 150 recognised genera (Rognes, 1991). They are distributed worldwide and exhibit a wide variety of larval feeding strategies ranging from carrion-breeding species to aggressive obligate parasites of livestock and wildlife. Thus, they are of great importance in a wide range of different disciplines and provide important ecosystem services (Putman, 1983).

The term blowfly is attributed to their carrion-feeding behaviour, as they often use decaying flesh for oviposition and larval development. It is said that meat is 'fly blown' or 'blown' when it has eggs laid on it. Their saprophagous behaviour has made them establish a close contact with humans since ancient times. For instance, in ancient Egypt, fly-shaped amulets were assigned to bodies during mummification process. It was thought that these amulets would return to the body whatever the flies would take away while feeding on the corpse (Kritsky, 1985). In some rural areas it is believed that some flies may carry the spirits of their departed ancestors (Kritsky, 1985). They also appear in important literature from Homer, Redi and even Shakespeare (Papavero et al., 2010).

1.2 Taxonomy

Within Calyptratae blowflies belong to the superfamily Oestroidea, which also includes bot flies (Oestridae), flesh flies (Sarcophagidae), tachinid flies (Tachinidae) and few other groups of flies (e.g. Rhinophoridae, Axiniidae, Mystacinobiidae).

The monophyly of Calliphoridae has been debated over decades (Tschorsnig, 1985; Rognes, 1991; Rognes, 1997). Using morphological features Rognes (1991) proposed the grouping of eight subfamilies within Calliphoridae: Calliphorinae, Chrysominae, Helicoboscinae, Luciliinae, Melanomyinae, Polleniinae, Rhiniinae and Rhinophorinae. However, a thorough analysis which used a maximum fit parsimony approach from forty-five adult and larval characters of 23 terminal monophyletic taxa within Oestroidea, conceived that the Calliphoridae are not a monophyletic group (Rognes, 1997). Certainly, the latter study suggests that the Rhinophoridae (woodlouse flies) cannot be treated as a subfamily within Calliphoridae (proposed by Rognes, 1986, 1991) neither as a sister group to Calliphoridae (proposed by Tschorsnig, 1985). Nevertheless, there is a growing body of research that has focused on the taxonomic study of the subfamilies Luciliinae (greenbottles), Calliphorinae

(bluebottles) and Chrysominae (screwworms) due to their economic, veterinary and forensic importance (Stevens and Wall, 1996; Stevens and Wall, 1997a; Stevens, 2003; Wallman et al., 2005; McDonagh and Stevens, 2011). The latter three groups are generally recognised as subfamilies within Calliphoridae (Rognes, 1997).

1.3 Life-cycle of blowflies.

Blowflies are holometabolous insects, which means they exhibit complete metamorphosis. The life-cycle starts from an egg, followed by the larval stage (LI, LII and LIII), pupariation and adult stage. As in almost all insects, blowfly rates of development are temperature-dependant, hence their life cycle is strongly affected by the temperature on which individuals are exposed (Wall et al., 1992b).

Although the life-cycle of individual species may vary according to the larval feeding behaviour, larval food source or temperature, the life-cycle of blowflies in general is very similar. Usually they lay batches of around 200 eggs. After hatching, larvae will start feeding on the dead or living tissue and continue to do so until they have completed 3 larval stages (Evans, 1936), which usually takes around 72 hours. Fully fed 3rd stage larvae migrate to the soil to begin pupation. Adults emerge after 3 days, depending on the temperature (Wall et al., 1992b). Once emerged, females need to feed on a proteinaceous substrate to produce fertile eggs (Wall, 1992). Females are ready to lay eggs usually after 3 days of emergence.

1.4 General importance of blowflies.

As previously mentioned, the different larval feeding strategies of blowflies have made them of great importance in many ways.

Firstly, their saprophagic behaviour gives them with a major ecological significance. Sarcosaprophagous flies are considered the principal invertebrate consumers of terrestrial carrion (Peschke et al., 1987). They perform an essential ecosystem service in the consumption, recycling and dispersion of carrion nutrients (Putman, 1983). Previous research has shown that they greatly reduce the carcass mass in both large and small vertebrate carcasses (Parmenter and MacMahon, 2009). Fig. 1.1 shows an elephant carcass found in Zambezi Valley, Zimbabwe, which 7-10 days after death, was consumed almost on

its entirety by blowfly larvae and other carrion-eating arthropods (Fig. 1.1b; M. Hall, personal communication).

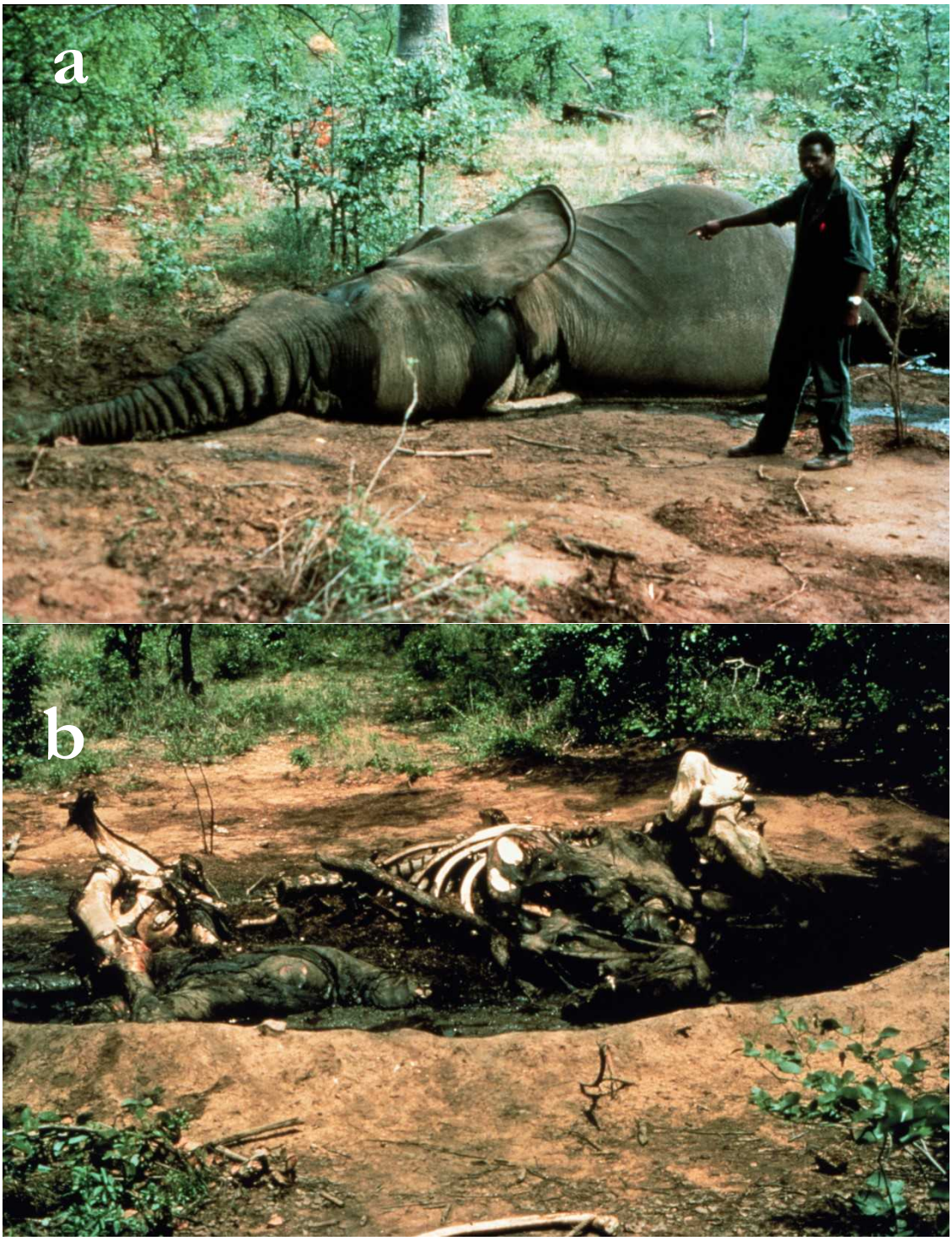

Figure 1.1 Elephant carcass found in the Zambezi Valley, Zimbabwe: a) Early stages of the carcass, person on the picture is Frederick Vakayi, a worker from the Rekomitjie Tsetse Fly Research Station. b) Elephant carcass after 7-10 days of decomposition, carrion was consumed almost entirely by blowfly larvae and other arthropods. Pictures were kindly provided by Martin Hall (Natural History Museum, London).

Carrion is an ephemeral resource which, unless very large, rarely allows blowflies to complete more than one generation in a carcass (Beaver, 1977). Thus, several species use the same resource which results in strong intra and interspecific competition (Hanski, 1987). Within the carrion-fly community niche differences allow the coexistence of similar species, including differences in their phenology, habitat and spatial distribution, carcass size, etc (Hanski and Kuusela, 1977; Smith and Wall, 1997; Martínez-Sánchez et al., 2001; Hwang and Turner, 2006). Knowing patterns of distribution and ecological differences of the carrion-fly community is of great importance for forensic entomologists (Greenberg, 1991; Hall, 2001; Zabala et al., 2014). Amongst carrion breeding arthropods, blowflies are considered as the most important group since they are found in greatest numbers and, usually, they are the first organisms to arrive and colonise a dead body (Hall, 2001). They can provide the most accurate information about the minimum time-since-death (Greenberg, 1991).

Studying the ecology of sympatric species could help our understanding of species co-existence, community assembly and dynamics of different groups that possess different or similar roles within an ecosystem (Pianka, 1999). Moreover, in evolutionary biology knowing the community dynamics, spatio-temporal variation and niche differentiation may also help answering evolutionary questions, such as the role of ecology in speciation (Pianka, 1999). Given the wide range of larval feeding strategies of blowflies, studying their ecological and behavioural differences is vital to explain their evolution and, in combination with molecular studies, it can provide robust information on the speciation and divergence of parasite and non-parasite lineages.

Over the course of evolutionary history, some calliphorid species have evolved ectoparasitic behaviour causing a diseases known as 'myiasis'. According to Zumpt (1965) myiasis is defined as *"the infestation of live human and vertebrate animals with dipterous larvae, which at least for a period, feed on the host's dead or living tissue, liquid body, substances, or ingested food"*. Some of them are of great economic significance as pests of livestock worldwide. For instance, *Lucilia sericata* is the main agent of ovine cutaneous myiasis or 'blowfly strike' in UK (Wall et al., 1992a; Hall and Wall, 1995). In 2004 sheep strike by the latter species affected around 72% of farms in Wales, Scotland and England (Bisdorff et al., 2006). Similarly, in Australia and New Zealand *Lucilia cuprina* (*dorsalis*) is the main agent of sheep myiasis and can generate considerable economic losses in sheep husbandry (Heath and Bishop, 1995; Tellam and Bowles, 1997). Economic losses in South-America due to the New World screwworm fly

(*Cochliomyia hominivorax* (Coquerel)) have been estimated to be over $3600 million per year (Vargas-Teran et al., 2005).

1.5 Evolution of myiasis.

Within the super family Oestroidea, there is a wide range of species that cause myiasis including blowflies (Calliphoridae), bot flies (Oestridae) and flesh flies (Sarcophagidae). It is clear that within Calliphoridae, the parasitic habit has evolved multiple times independently (Stevens and Wall, 1997a; Stevens and Wallman, 2006; Stevens et al., 2006). Myiasis agents can be divided in different categories according to the host-parasite interaction (Zumpt, 1965)(Table 1.1). Phylogenetic analyses have highlighted the close relationships held between parasitic and non-parasitic sister taxa (Stevens, 2003). Understanding their evolutionary relationships can provide broad insights to the origin of parasitism in true flies. It has been speculated that these distinct behaviours may be mediated by differences in temperature tolerance, response to the immune system of the host and the production/secretion of enzymes associated with feeding on the host tissues (Stevens and Wallman, 2006). Certainly, the first larval stages of primary myiasis agents are able to induce local inflammatory responses in their host, and accompanied by a damage to epidermal cells and larval proteolytic enzyme secretion they work to initiate the wound development on the skin of their host (Sandeman et al., 1985; Sandeman et al., 1987).

1.5.1 Oestridae.

Botflies (Oestrid flies) are true obligate parasites that exhibit very high host-specificity and relatively low pathogenicity (Table 1.2). They are usually endoparasites with relatively ancient associations with the host (Stevens et al., 2006). They can cause myiasis in internal organs of the host (*Hypoderma* spp.), nasopharyngeal tracts (*Oestrus* spp.), digestive tracts (*Gasterophilus* spp., Fig. 1.2) and subcutaneous tissue (*Przhevalskiana* spp.)(Otranto et al., 2003). Their larval feeding period can last for several weeks or even months within their host (Pape, 2006). These obligate larvae produce proteases that enable them to survive the immune system of the host. For instance, the first larval stages of *Hypoderma* secrete serine proteases (chymotrypsin and hypodermin) that will not only assist them in combating the immune response of the host, but also facilitates the larval migration and movement within the host (Chaubadie and Boulard, 1992; Boulard et al., 1996; Otranto et al., 2003).

Table 1.1 Classification of myiasis according to their relationship with the host. Source: Zumpt (1965)

Group	Description	Examples
Obligate	Dependent on their host for at least one stage of their life-cycle.	Botflies (Oestridae)
Facultative – Primary	Do not depend on their host to complete their life cycle, however they are able to initiate myiasis.	*Lucilia sericata*, *Lucilia cuprina* (Callophoridae)
Facultative – Secondary	Do not depend on their host and are unable to initiate myiasis. However, might be involved in myiasis once the wound has already been initiated by a primary agent.	*Calliphora vicina* (Calliphoridae)
Accidental	May cause pathological reactions due to accidental ingestion.	*Sarcophaga* spp. (Sarcophagidae)

It has been suggested that this group of flies could have evolved originally from rodent parasites (Pape, 2001) Recent mitogenomic studies indicate that the main diversification of Oestridae began with the wide radiation of mammal fauna that occurred during the Paleogene (Junqueira et al., 2016). Certainly, parallel evolution could have played a role in speciation of Oestridae by parasite lineages tracking host lineages through evolutionary time (Stevens and Wallman, 2006).

Figure 1.2 Ventral view of a larval sample of a horse bot-fly (*Gasterophilus* sp.). Picture taken at the Veterinary Parasitology collection at University of Bristol, Life Sciences Building.

1.5.2 Sarcophagidae

Generally, flesh flies are ovolarviparous insects. This means the egg development and hatching occurs within the female. They exhibit different feeding larval behaviours, ranging from saprophagous, coprophagous, parasitoids and parasites (Pape, 1996). Unlike Oestridae, Sarcophagids have low host-specificity and larvae development occurs in a shorter period of time often with high pathogenicity (Table 1.2). The most representative myiasis-causing species of this group is *Wohlfartia magnifica* (Schiner), which is an obligate parasite and an important pest of sheep in Southern Europe including Spain, Hungary, Bulgaria and Romania (Sotiraki et al., 2010), however it has been reported causing myiasis in humans, pigs, horses, camels and dogs (Hall and Farkas, 2000).

1.5.3 Calliphoridae

Parasitic lineages of Calliphoridae exhibit many different forms of parasitism (Table 1.2), ranging from highly specialised obligate parasitism (e.g. *Lucilia bufonivora*) to an opportunistic facultative parasitism, for example *Calliphora vicina* (Robineau-Desvoidy) (Zumpt, 1965). Nonetheless, the majority exhibit low host-specificity. As in the Sarcophagidae, larvae development and myiasis occurs in shorter periods of time and with high pathogenicity (Stevens and Wallman, 2006; Stevens et al., 2006). Due to the life history of this group, it has been hypothesized that the parasite lineages may have had saprophagic origins and that the parasitic behaviour in some species evolved in association with humans and animal domestication (Erzinclioglu, 1989; Stevens and Wall, 1997a)

Several species of this group exhibit primary facultative parasitism. Some of these species have a significant economic impact as pests of livestock. For example *Lucilia sericata* and *Lucilia cuprina* (Wiedemann) behave as the main sheep myiasis agents in Northern Europe and Australia respectively. Some species might exhibit secondary facultative parasitism and occasionally will be involved in myiasis once the wound has already been initiated by a primary agent, such as species of the genera *Calliphora*, *Phormia* and *Protophormia*. Usually secondary myiasis agents are not of great economic concern (Zumpt, 1965).

This group also includes species that exhibit obligate parasitism, however the nature of their host-parasite relationship, differs greatly to that seen with Oestrid flies. They usually have considerably higher pathogenicity (Stevens et al., 2006). This has made them of great concern as pests of livestock in many different parts of the world. For example, in tropical and sub-tropical areas of the Western hemisphere, the New world screwworm fly *C. hominivorax* (Fig. 1.3) is considered one of the most destructive insect pests of livestock (Klassen and Curtis, 2005; Vargas-Teran et al., 2005). Similarly, in the Eastern hemisphere, the Old world screwworm fly, *Chrysomya bezziana* (Villeneuve), occupies equivalent ecological niches to *C. hominivorax* and is also an important pest of livestock (Zumpt, 1965).

Figure 1.3 Lateral view of a larval sample of the New world Screwworm fly *(Cochliomyia hominivorax*). Specimen kept at the Veterinary Parasitology collection at University of Bristol, Life Sciences Building.

To a lesser extent some species have evolved a highly specialised obligate parasitism. The genus *Protocalliphora* is composed by species that are blood-feeding obligate parasites of birds (Whitworth and Bennett, 1992). Similarly, two species of the genus *Lucilia* are known to be highly specialised obligate parasites of amphibians, *L. bufonivora* and *Lucilia elongata* (Brumpt, 1934; Zumpt, 1965). The latter two species are of particular evolutionary interest, as they are probably the only two species that exhibit highly specialised obligate parasitism within a genus that comprises mostly saprophagous and facultative species.

Table 1.2 Myiasis in Oestroidea. The taxonomic family, species name, host interaction, specificity and range of the different taxa known as myiasis agents. Sources: Zumpt(1965); Rognes(1991); Wall et al. (1992a); Vergas-Teran et al. (2005); Pape(2006); Sotiraki(2010); Tantawi and Whitworth(2014).

Family	Name	Host- parasite interaction	Host-specificity	Host Range	Distribution
Oestridae (bot flies, warble flies)	*Dermatobia hominis*	Obligate	High	Primates	South-America
	Hypoderma bovis	Obligate	High	Cattle	Holarctic
	Oestrus ovis	Obligate	High	Ovine livestock	Widespread
Sarcophagidae (flesh flies)	*Wohlfartia magnifica*	Obligate	Low	Mammals. Main host: Sheep	Mediterranean. Main agent of sheep myiasis in Spain.
Calliphoridae (blowflies)	*Lucilia sericata*	Facultative. (Primary)	Low	Main host: Sheep	Widespread, primary sheep myiasis agent in UK.
	Lucilia cuprina	Facultative. (Primary)	Low	Main host: Sheep	Widespread. Primary sheep myiasis agent in Australia, New Zealand and South-Africa
	Lucilia caesar	Facultative. (Secondary, although it might act occasionally as primary)	Low	Main host: Sheep	Widespread. Occasionally involved in sheep strike cases of UK.
	Lucilia illustris	Facultative (Secondary)	Low	Main host: Sheep	Widespread. Commonly involved in sheep strike in Norway
	Lucilia bufonivora	Obligate	High	Amphibians	Europe, Asia and Canada
	Cochliomyia hominivorax	Obligate	Low	Mammals. Main host: Cattle	South-America
	Chrysomya bezziana	Obligate	Low	Mammals Main host: Cattle	Tropical and Subtropical Asia and Africa.

1.5.4 Evolution of ectoparasitism in the genus *Lucilia*

Lucilia (Diptera:Calliphoridae) is a relatively homogeneous group of blowflies, known as greenbottles, that include at least 27 species distributed throughout the Holoarctic occurring in all faunal regions (Rognes, 1991). Although they display a range of larval feeding strategies, they bear a close morphological resemblance to each other (Zumpt, 1965; Rognes, 1991).

It has been hypothesised that parasitic behaviour has evolved multiple times independently within *Lucilia,* probably in association with humans and animal domestication (Erzinclioglu, 1989; Stevens and Wall, 1997a). This parasitic behaviour is variable within and between different species of *Lucilia*. In Northern Europe the sheep blowfly *L. sericata* is the main agent of ovine cutaneous myiasis, usually known as 'flystrike' (Table 1.2). It is a facultative ectoparasite of great economically concern in sheep-producing farms, especially in UK (MacLeod, 1943; Wall et al., 1992a). Although its distribution and behaviour overlap with *L. cuprina* in many parts of its range, the latter occupies the equivalent ecological niche as the main sheep myiasis agent in warmer countries such as, Australia and New Zealand (Heath and Bishop, 1995; Tellam and Bowles, 1997).

The British and Australian sheep blowflies, *L. sericata* and *L. cuprina* respectively, comprise a homogeneous group of species within *Lucilia* that are extremely similar in morphology. One of the main morphological characters that define them is the pale basicosta and the 3 pairs of post-acr bristles (Aubertin, 1933; Rognes, 1991). This group also includes species that are considered rare due to their low abundance, such as *Lucilia richardsi* (Collin), *Lucilia regalis* (Meigen) and *Lucilia pilosiventris* (Kramer). In the Palearctic, *L. richardsi* is sympatric with *L. sericata* in many parts of its range. They are extremely similar morphologically and phylogenetically (Aubertin, 1933; Stevens and Wall, 1997a). However, despite their close relationships, there are no existing records of the involvement of *L. richardsi* in ovine cutaneous myiasis and its biology is poorly known. Nuorteva and Skarén (1960) noted that it is strongly attracted to small carcasses of homothermal animals like birds and small mammals, and there is only one record its involvement in a wound myiasis of a nightjar (Nuorteva, 1959). Similarly, *L. regalis* and *L pilosiventris* are rare species for which little of their biology is known. Based on morphological characters Stevens and Wall (1996) highlighted their close relationship with *Lucilia silvarum* and *L. cuprina*. There are, however, no detailed phylogenetic studies that explore their relationships and taxonomic position within the genus *Lucilia* using molecular tools.

Lucilia caesar, *Lucilia illustris* (Meigen) and *Lucilia ampullacea* (Villeneuve) comprise a group of mainly saprophagous species that are also morphologically and genetically very similar (Stevens and Wall, 1996; Stevens and Wall, 1997a). They are well differentiated from the *L. sericata* species group by the presence of a black basicosta and 2 post acr bristles (Rognes, 1991). Although rarely found as primary agents of myiasis, *L caesar* may be involved in myiasis in Northern Europe (Brinkmann, 1976; Wall et al., 1992a). *L. illustris* has been reported as a common species involved in ovine myiasis in Norway (Brinkmann, 1976), and in Finland it is the dominant species of the carrion-fly community (Hanski and Kuusela, 1977).

A few species exhibit an extremely specialised obligate form of parasitism for amphibians, such as the toad fly, *L. bufonivora* (Brumpt, 1934). Its life history suggest that the evolution of obligate parasitism occurred independent of human activity and had very different origins to the ones exhibited by the sheep blowfly. Due to the low economic impact of *L. bufonivora*, its taxonomy, biology and evolutionary history have been poorly studied. To date, the forces that have driven this highly specialised behaviour are poorly understood.

1.6 Amphibian myiasis

Unlike sheep myiasis, the mortality rate of amphibians infested with blowfly larvae is very high and usually do not survive the infestation (Brumpt, 1934). In Europe two species are thought to be involved in this disease: *L. silvarum* and *L. bufonivora* (Duncker, 1891; Mortensen, 1892; Linder, 1924; Stadler, 1930; Rognes, 1991). The former species is considered of forensic importance, as it has been found breeding in carrion and it is a common species of the carrion-fly community of Finland (Hanski, 1987; Fremdt et al., 2012). There is, however, no existing record of *L. bufonivora* breeding in carrion, which highlights its behaviour as an obligate parasite. Although there are reports of *L. silvarum* involved in amphibian myiasis, some authors argue that they might have been product of misidentification with *L. bufonivora* (Zumpt, 1965). Certainly, larval morphological identification is nearly impossible, and the adult stages share many morphological features (Rognes, 1991). Usually identification is done with the number of post acr bristles (2 and 3 in *L. bufonivora* and *L. silvarum* respectively)(Aubertin, 1933). However, Rognes (1981, 1991) notes that this feature is not completely reliable because the number of bristles can be very variable. DNA-based identification is rarely performed to enable their differentiation. Thus,

to date, the species composition of *Lucilia* involved in amphibian myiasis in Europe is not well understood.

1.6.1 Life-Cycle

Females of the toad fly lay eggs on the surface of their host, often scattering them on the back of their host. After hatching first instar larvae migrate to the nasal cavities of their host where they start larvae development and feeding (Brumpt, 1934). Some reports of the pathology of amphibian myiasis in the North American continent differ slightly to the ones from Europe. In North America, amphibian myiasis reports have described myiasis wounds in the hind legs and back of the host (Bolek and Coggins, 2002; Bolek and Janovy, 2004). Whether this is a genuine difference or incidental variation is not known.

As with most calliphorid flies, they pass through three larval stages. This process often kills the host after which the larvae may remain feeding in the carcass for a short period of time (Brumpt, 1934). Once the larvae are fully developed, they migrate to the soil, where they undergo pupariation. Although it is not yet clear, the absence of adults during colder months suggest that they overwinter through larval diapause, which is a common feature in *Lucilia* blowflies (Pitts and Wall, 2005). In northern regions adult flies can be found from June to August (Rognes, 1991).

1.6.2 Host range.

It has been assumed that in Europe the most common host for *L. bufonivora* is the common toad, *Bufo bufo* (Linnaeus) (Strijbosch, 1980; Weddeling and Kordges, 2008; Martín et al., 2012). However, this needs to be confirmed using molecular tools because morphological identification of larvae is extremely difficult. Regardless, amphibian myiasis is not restricted to the common toad, in fact it has been recorded as affecting a wide range of amphibian hosts including *Salamandra salamandra* (Linnaeus), *Rana temporaria* (Linnaeus) and *Epidalea calamita* (Laurenti) which is an endangered species in the UK (Brumpt, 1934; Vestjens, 1958; Koskela et al., 1974; Weddeling and Kordges, 2008; Gosá et al., 2009).

1.6.3 Amphibian myiasis in North America

In North America two species of *Lucilia* are thought to be involved in amphibian myiasis: *L. silvarum* and *L elongata.*(Bolek and Coggins, 2002; Bolek and Janovy, 2004). The latter species has never been reported breeding in carrion, therefore it is also considered an obligate parasite of amphibians. In this range, amphibian myiasis is reported affecting various frog species more often than it is in Europe. These include Nearctic species such as the wood frog, *Lithobates sylvaticus* (LeConte) and the western chorus frog *Pseudacris triseriata* (Wied-Neuwied) and bufonids like the western toad *Anaxyrus boreas* (Baird and Girard) and the American toad *Anaxyrus americanus* (Holbrook)(James and Maslin, 1947; Roberts, 1998; Bolek and Janovy, 2004; Eaton et al., 2008).

It was thought that *L. bufonivora* was restricted to the Palaearctic, however Tantawi and Whitworth (2014) made the first report of this species in Canada. As in Europe, this study also reported that misidentification between *L. silvarum* and *L. bufonivora* is very common. Certainly, the North American keys by Hall (1948) do not include *L. bufonivora*, and include only *L. silvarum* and *L. elongata* (which is listed as '*Bufolucilia silvarum*' and '*Bufolucilia elongata*' respectively). This is unfortunate because Hall's keys have been used widely for the identification of flies reared from diseased amphibians (Bolek and Coggins, 2002; Eaton et al., 2008). Thus, the reports of *L. silvarum* causing amphibian myiasis in North America are also ambiguous. So far no molecular studies have been performed to determine the amphibian myiasis species composition in North America, and there are no existing studies of the evolutionary relationships of this species group. Moreover, it is not known whether the toad fly *L. bufonivora* has been present in North America since relatively ancient times or whether it was only recently introduced.

1.7 Molecular systematics of blowflies

In the past two decades there has been a significant increase in the use of molecular tools for phylogenetic inference and identification of blowflies (Wallman et al., 2005; Junqueira et al., 2016). DNA-based diagnostics have proved to be particularly useful for the identification of larval specimens or damaged specimens of which morphological identification is ambiguous. Furthermore, molecular tools are of vital importance in forensic entomology (Yusseff-Vanegas and Agnarsson, 2017). Use of DNA sequence data for phylogeny inference and analysis has helped on solving taxonomic problems from different groups within Calliphoridae (Stevens, 2003; Wallman et al., 2005; Williams et al., 2016).

DNA-based methods do, however, have some disadvantages. For instance, using single-locus approaches as an identification source is not completely reliable. A barcoding approach often fails to identify recently diverged taxa and/or closely related species (Nelson et al., 2007; Whitworth et al., 2007). Thus, multi-gene approaches are needed to give stronger reliability for unambiguous identification and phylogeny inference (Wallman et al., 2005; McDonagh and Stevens, 2011)

1.7.1 Mitochondrial markers.

Mitochondrial DNA (mtDNA) has been widely used for blowfly phylogenetics and identification (Otranto and Stevens, 2002). It has several advantages over other molecular markers. Some of these are, for instance, the lack of recombination and the high copy number which makes it easy to isolate and amplify. This markers exhibit both conserved and variable regions and it is relatively easy to access universal primers for amplification (Avise et al., 1979; Folmer et al., 1994; Lunt et al., 1996). Furthermore, mtDNA usually exhibit much higher mutation rates than nuclear DNA (Brown et al., 1979), which makes it particularly useful for inferring relationships of recently diverged taxa (Stevens and Wall, 1997b; McDonagh and Stevens, 2011). Amongst mtDNA markers, Cytochrome oxidase subunit I (COXI) and Cytochrome-b (Cyt-B) have been used widely for blowfly phylogenetics (Wallman et al., 2005; McDonagh and Stevens, 2011; Yusseff-Vanegas and Agnarsson, 2017). Mitochondrial markers have also proved useful for inferring divergence times within taxa (Wallman et al., 2005).

1.7.2 Nuclear markers.

Nuclear DNA (nDNA) possess several advantages such as the presence of exons and introns and also a low bias level of base composition (Brower and DeSalle, 1994; Friedlander et al., 1994; Lin and Danforth, 2004). Usually these markers have greater power for resolving deeper node levels (Baker et al., 2001). However, nDNA has a lower copy number than mtDNA, which could result in a relatively more difficult PCR amplification. There is also the risk of occasional occurrence of paralogous loci (Lin and Danforth, 2004).

Some of the nDNA markers that have been used for insect systematics, to mention a few, are elongation factor-1 alpha (*EF1- a*)(McDonagh and Stevens, 2011), dopa decarboxylase (*DDC*)(Tatarenkov et al., 1999), phosphoenolpyruvate carboxykinase (*PEPCK*)(Wiegmann et al., 2000). Moreover, recently developed nuclear markers have been used for the detection

of hybrids between the closely related species of sheep blowflies (*L. cuprina* and *L. sericata* respectively) in South Africa (Williams and Villet, 2013)

1.7.3 Non-Coding ribosomal DNA: *ITS2*

Non-coding ribosomal DNA, such as the Internal transcribed spacer two (*ITS2*), often exhibit higher mutation rates than mtDNA (Otranto and Stevens, 2002). This gene is transcribed as a larger precursor RNA molecule that contains three different ribosomal RNA (rRNA) subunits (18S, 5.8S and 28S) plus two internal transcribed spacers (ITS1 and *ITS2*) and two external transcribed spacers (5'-ETS and 3'-ETS)(Hillis and Dixon, 1991). A variety of structures that are created by the folding of the peptide chain form the secondary structures of the rRNA molecules. These secondary structures are usually conserved among taxa and make it a suitable marker for phylogenetic inference (Marinho et al., 2011). It has a high copy number and PCR amplification and sequencing are relatively easy. Recent studies has shown that *ITS2* is a suitable marker for phylogenetic analyses at both species and generic levels (Marinho et al., 2011; Yusseff-Vanegas and Agnarsson, 2017).

1.8 Aims

The work described in this thesis had four primary aims. This first was to undertake an analysis of niche differentiation among the species of *Lucilia* found in the UK, to consider factors that facilitate their coexistence in the field. The second was to determine the species composition in amphibian myiasis in Europe from unidentified larvae of toad-myiasis cases using DNA-based identification methods. The third aim was to consider the taxonomy and ecology of the toad fly, *L. bufonicora*, using both molecular phylogenetics and field studies of its ecology to help to resolve its status as a species. Finally, to undertake a broader phylogenetic analysis to understand evolution of obligate amphibian parasitism within the genus *Lucilia*.

2. Spatial and temporal habitat partitioning by calliphorid flies

Contributions

Gerardo Arias-Robledo was the main researcher and undertook all sample collection, identification as well as statistical analysis and wrote the drafts of the manuscript. Richard Wall assisted as the main supervisor and supported with manuscript corrections and interpretation of results. Jamie Stevens supported with corrections the manuscript. Charlotte Chivers supported as a field assistant. Mike Amos, Paul Amos and Andrew Hughes provided access to trapping sites. The work was funded by CONACyT and University of Bristol and it was published in the journal **Medical and Veterinary Entomology** (see **appendix I**).

2.1 Introduction

Carrion-breeding arthropod fauna, which incorporates blowflies in general, perform an essential role in an ecosystem, ensuring the consumption, dispersion and recycling of carrion nutrients (Putman, 1983; Parmenter and MacMahon, 2009). Carrion is an ephemeral resource which rarely allows them to complete more than one generation in a carcass (Beaver, 1977). Thus, several species use the same resource which results in strong intra and interspecific competition (Hanski, 1987).

Within the carrion-fly community niche differences allow the coexistence of similar species, including differences in their phenology, carcass size, synanthropy, etc (Hanski and Kuusela, 1977; Smith and Wall, 1997; Hwang and Turner, 2006). Habitat and species phenology may have a very important role on the species segregation of the blowfly community. For instance, *Calliphora* species are more abundant in early seasons and colder months, as they have a lower temperature requirement (Greenberg, 1991). *Lucilia* blowflies, however, have higher temperature requirements which results in an effective temporal segregation between *Lucilia* and *Calliphora* species (Greco et al., 2014; Zabala et al., 2014).

MacLeod and Donnelly (1956) recorded thirteen different species from the carrion-blowfly community of UK (not counting *Lucilia bufonivora*). Six species of *Lucilia*, five species of *Calliphora*, one species of *Protophormia* and one species of *Cynomya* . A more recent study of the necrophagous fly community of South-east England (Hwang and Turner, 2006), have also found 3 species of *Pollenia* and one species of *Melinda.* The latter study found *Calliphora vicina* the most abundant species of the carrion-fly community. In the South-West, *Lucilia sericata*, *Lucilia caesar* and *C. vicina* are amongst the most common species that emerge from carcasses in the field (Smith and Wall, 1997b).

The sheep blowfly (*L. sericata*) is a cosmopolitan species distributed throughout the world (Aubertin, 1933; Hall, 1948; Rognes, 1991). It is usually confined to open and exposed habitats (Gregor, 1991; Smith and Wall, 1997; Martínez-Sánchez et al., 2001). Woodridge et al. (2007) showed that the catch size of this species is significantly affected by light intensity. It is a synanthropic species, frequent in urban and populated areas (Fischer, 2000; Hwang and Turner, 2006). In Northern Europe, adult flies are found from April to October, usually in higher abundancies during the warmer months (Rognes, 1991).

On the other hand, *L. caesar* is usually more abundant in shaded rather than open habitats (MacLeod and Donnelly, 1956). *Lucilia illustris* is a very rare species in UK but very common

in Finland(Hanski, 1987). Macleod and Donelly (1956) noted that, unlike *L. caesar*, this species occurs in a wide range of habitat types, including shaded and non-shaded. The behaviour *Lucilia ampullacea* resembles to the one of *L. caesar*, however it exhibits a stronger confinement to shaded habitats and it is almost never recovered from open habitats (MacLeod and Donnelly, 1956).

This aim of the work described in this Chapter was to examine the structure of a calliphorid community on farmland in south west England and to determine the roles of habitat, temperature and farm type on its species composition and its spatial and temporal abundance.

2.2 Materials and methods

2.2.1 Study sites

Two sites were sampled in this study. Site one was on an organic livestock farm in Failand, North Somerset, situated in a rural area consisting mainly of pastureland for agricultural use and with little human activity (Fig. 2.1). Site two was on a mixed farm in Long Ashton, North Somerset, located in a semi-rural zone less than 500m away from an area with housing and higher human activity (Fig. 2.2). Each farm was situated approximately 5km from the city of Bristol and separated by 5km from each other. Both sites presented a variety of different patches of habitat within the farm, of which three were considered for this study: open field (grassland, with direct sunlight, Fig. 2.3a), hedgerow (mainly hawthorn and bramble, offering partial shade, Fig. 2.3b) and woodland (predominantly ash, hazel and birch, with complete shade, Fig. 2.3c).

Figure 2.1. Rural site with little human activity situated in North Somerset, England.

2.2.2 Trapping

Modified bottle traps (Hwang and Turner, 2006) were used to catch blowfly specimens in good condition to allow identification (Fig. 2.3d). Each trap was baited with approximately 100g of fresh lamb liver. The liver was placed in a plastic container inside the trap and it was topped up with water during every collection to prevent bait desiccation. The bait was covered with a mesh and a rubber band to reduce oviposition.

On the 2nd of March 2016, five traps were distributed at least 20 m from each other in each habitat placed approximately 1.5 m off the ground and attached to a tree, for the woodland, or a fence post for the hedgerow. To sample in open habitats, pre-existing posts supporting wire fences with no associated hedge vegetation were used or, if unavailable, wooden posts were erected for the study prior to trapping. Fifteen traps were placed at each farm, five in each habitat with a total of 30 traps for both farms. The last collection and trap removal took place on the 5th of August 2016.

Figure 2.2. Semi-rural farm with moderate human activity situated in North Somerset, England.

Collections were made every 3-6 days. At each collection, the upper part of the trap containing the flies was removed and replaced by a clean one for further trapping. Traps were taken back to the laboratory at the University of Bristol and placed at -20 °C for 20 min to kill the flies. Traps were then emptied and specimens were removed for further identification. Baits were replaced every 4-5 weeks; previous research has shown that the age of the baits has little effect on the blowfly catch size once past the initial stages of decomposition (Fisher et al., 1998). Once trapped flies had been removed, calliphorids were separated from non-target species and identification was made under a dissecting microscope model Leica S6E using keys (Emden, 1954). The number of each species was recorded in relation to habitat and site.

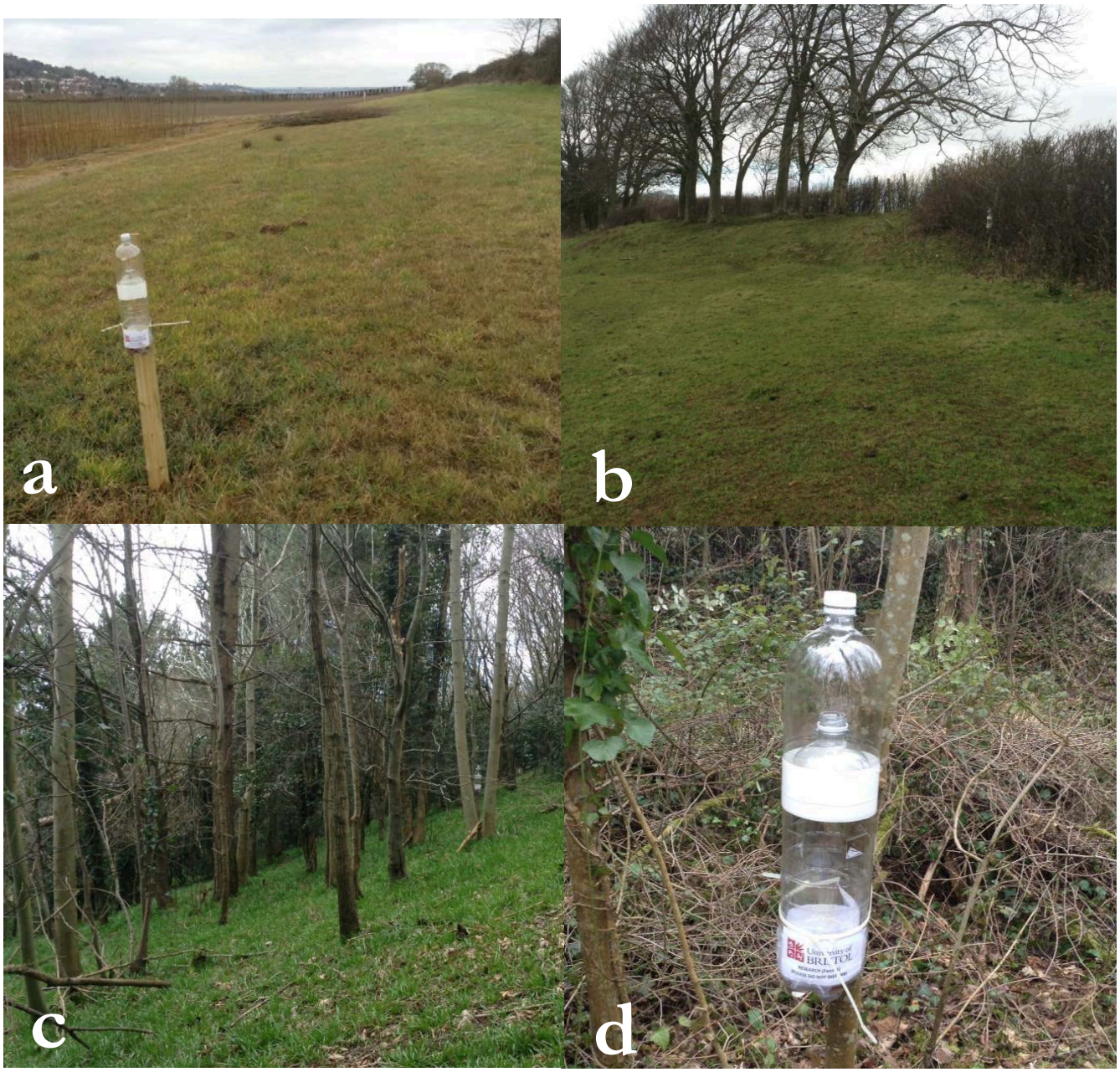

Figure 2.3. Habitats sampled in this study: open (a), hedgerow (b) and woodland (c). Picture d) displays the modified trap used for blowfly sampling. Bait was placed in the bottom part of the trap and the upper part held the trapped flies until collection was done. Pictures a) and c) were taken at the semi-rural site, where as pictures b) and d) were taken at the rural site.

2.2.3 Data analysis

The number of flies caught per trap per day was calculated by dividing the number of flies caught per trap (for individual species) by number of days of trap operation since the last collections. Catch per day was used to remove any effect of the differences in time interval between collections. For each collection interval, mean temperature was calculated from the data recorded by a local weather station at Horfield/Filton (Table 2.1), situated approximately 13 km from both farms. Due to the non-normal distribution of the count data for the calliphorid species collected here (Fig. 2.4) a generalised linear mixed model with a negative binomial error was selected for each species separately with the function glm.nb using R in RSTUDIO 3.4.2 (2015) where the influence of site (rural, semirural), habitat (open, hedge, woods) and temperature were included as fixed factors and the transformed fly count number (described above) as the dependant variable. Previous studies have shown that for overdispersed count data, negative binomial distribution models can provide better understanding of the probability distribution of different species (Sileshi, 2006). The best fit model was selected by the stepwise removal of non-significant factors for each separate species based on the Akaike information criterion AIC (Table 2.2). If any, interactions between site and habitat were also analysed.

2.3 Results

This work confirmed the presence of nine Calliphorid species in South-West UK: *Lucilia richardsi*, *Lucilia silvarum*, *L. sericata, L. caesar*, *L. illustris*, *L. ampullacea*, *Calliphora vomitoria* (Linnaeus), *C. vicina*, *Cynomya mortuorum* (Linnaeus) and *Protophormia terranovae* (Robineau-Desvoidy). A total of 17,246 calliphorid specimens were caught and identified. Of these 2,427 were *L. sericata*, 51 *L. richardsi*, 6,580 *L. caesar*, 307 *L. ampullacea*, 4,881 *Calliphora vicina* and 2,959 *C. vomitoria*. Least abundant species like *C. mortuorum*, *P. terranovae*, L. *silvarum* and *L. illustris*, were not included in the statistical analysis, as the number of specimens caught for each was less than 10. No specimens of *Lucilia bufonivora* were caught during this study.

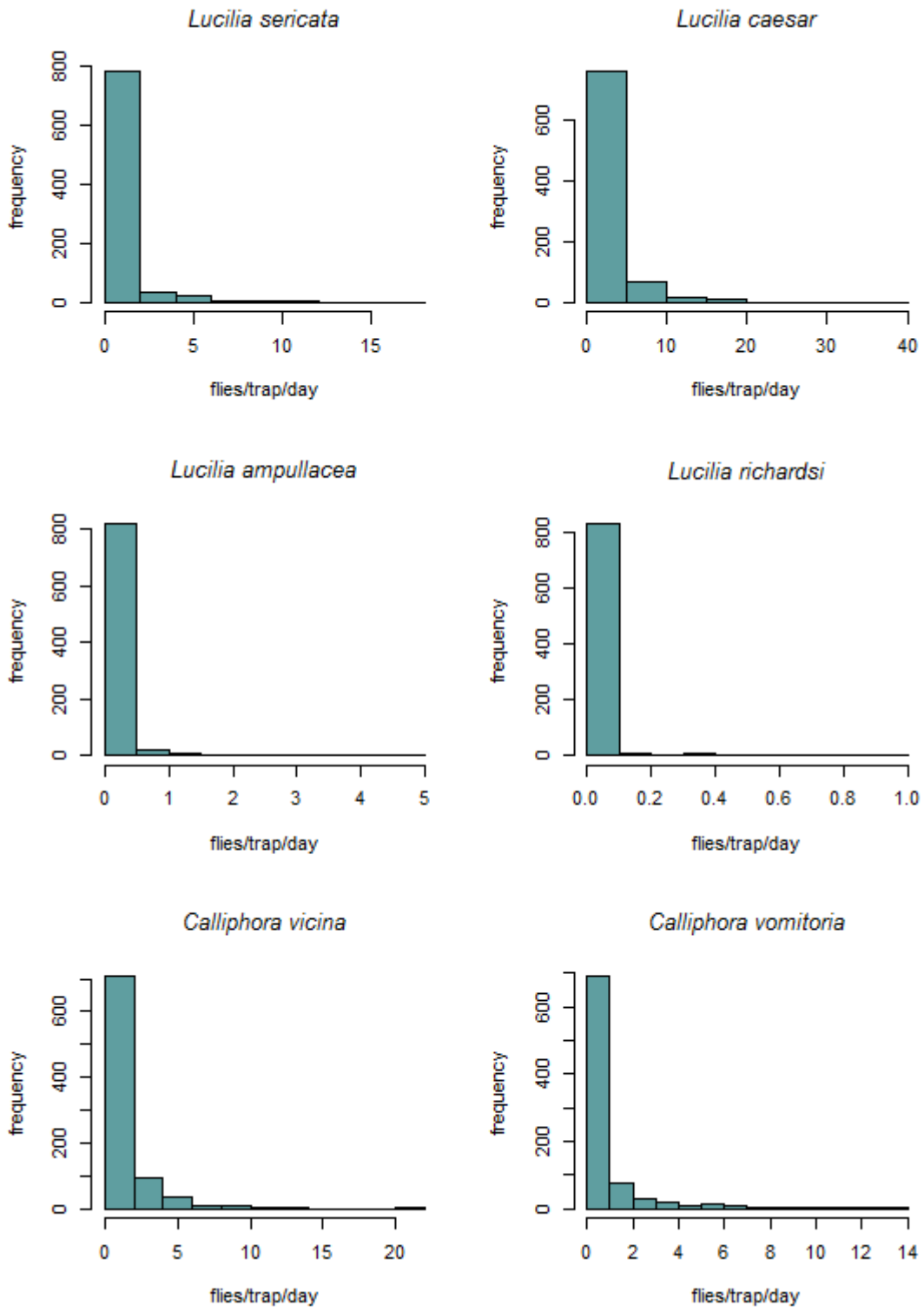

Figure 2.4 Distribution frequencies of the calliphorid species recorded in this study. *X* axis displays the flies/trap/day and *Y* axis represents the frequency. Individual species names are indicated at the top of their respective histogram plot.

2.3.1 Model Selection

Models selected for *L. sericata*, *L. caesar,* and *C. vomitoria* included habitat, site, temperature and the interaction between the latter 2 factors (Table 2.2). The model selected for *L. ampullacea* removed interactions between 'site' and 'habitat' as they were not significant (Table 2.2). *L. richardsi* was the least abundant of the 6 species and in order to analyse the habitat distribution of this rare species, the model selected did not include the interactions between habitat and site. The best fit model for *C. vicina* did not include 'temperature' and just included 'site', 'habitat' and their interactions, resulting in lower AIC scores (Table 2.2).

Calliphorid species composition changed over the duration of the collection period. *C. vicina* and *C. vomitoria* were the first calliphorid species to emerge in the month of March when the average temperature was 6.8 °C. No *Lucilia* specimens were found in March (Fig. 2.5). The two *Calliphora* species were also the most abundant calliphorids over the month of April; the average temperature reported for this month was 8.9 °C (Table 2.1). In fact the highest catch recorded for the month of April of *C. vicina* was of 12 flies/trap/day. The first specimens of *L. sericata* and *L. caesar* were observed during late April at the semirural farm (Fig. 2.8, Fig. 2.9). The population of *C. vicina* decreased in the warmer months, June, July and August, while *Lucilia* populations increased over these months (Fig. 2.5). The statistical models showed that there was a significant effect of temperature on the number caught for all calliphorid species except for *C. vicina*, where temperature was not a significant factor (Table 2.2). Nonetheless, Fig. 2.5 shows the early emergence of *C. vicina* showing strong segregation between it and *Lucilia caesar*.

Table 2.1. Seasonal temperatures recorded in Bristol (Horfield/Filton weather station) for each sampling month (March-August 2016). Month of study, average temperature, average maximum temperature and average minimum temperature are displayed in the table.

Month	Average temperature (°C)	Average max temp (°C)	Average min temp (°C)
March	6.8	10.2	3.4
April	8.9	13.2	5.1
May	13.9	18.6	9.5
June	16.2	20.2	13.1
July	17.7	21.7	14.1
August	16.6	20.9	13

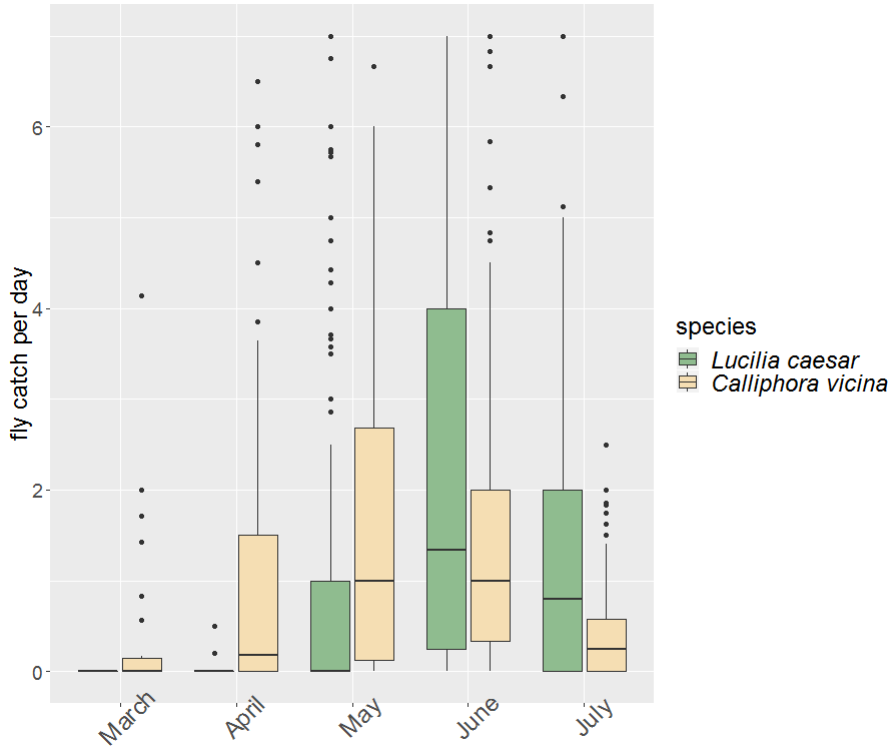

Figure 2.5. Median number of flies caught (trap/day) recorded for two farms in different months; boxes represent first and third quartile for *Lucilia caesar* and *Calliphora vicina*. Whiskers show 95% confidence intervals with outliers (dots).

Table 2.2. Stepwise removal of non-significant factors for individual species models. Table displays the formula used, its degrees of freedom and its AIC scores. *=model selected for statistical analysis of individual species.

Model	df	*L. sericata*	*L. caesar*	*L. ampullacea*	*L. richardsi*	*C. vicina*	*C. vomitoria*
glm.nb(nflies~1)	2	3401.58	5204.87	1193.25	378.011	5552.66	4014.88
glm.nb(nflies~temp)	3	3294.63	5002.32	1154.45	375.644	5550.61	3986.53
glmer.nb(nflies~site*habitat)	7	3211.52	5097.14	1045.01	345.421	5414.74*	3656.36
glmer.nb(nflies~site*habitat+temp)	8	3087.54*	4847.13*	1003.15	340.349	5416.74	3619.60*
glmer.nb(nflies~site+habitat+temp)	6	3102.83	4852.61	999.788*	341.727*	5426.90	3620.65

2.3.3 Effects of site

Of the 17,246 specimens, 7,876 flies were caught in the rural farm and 9,370 in the semi-rural farm. Site had a significant effect on the abundance of *L. sericata* ($z=7.142$, $P<0.001$; Fig. 2.6) and *L. ampullacea* ($z=-9.591$, $P<0.001$; Fig. 2.6); however, this factor had no association with the abundance of *L. caesar, L. richardsi, C. vicina* or *C. vomitoria* (Table 2.2). The sheep blowfly *L. sericata* was significantly more abundant at the semi-rural farm than in the rural farm (Fig. 2.6). In contrast *L. ampullacea* was significantly more abundant at the rural farm (Table 2.3, Fig. 2.6).

Table 2.3. Effects of site on individual species abundance. Table displays the estimate, standard error, z value and p values computed by the model for each calliphorid species studied.

Factor	sp.	estimate	SE	z	p
Semirural - Rural	*L. sericata*	1.9526	0.2734	7.142	<.0001
Semirural - Rural	*L. caesar*	0.3119	0.2308	1.351	0.1767
Semirural - Rural	*L. ampullacea*	-0.9815	0.2897	-3.388	<.0001
Semirural - Rural	*L. richardsi*	0.7394	0.555	1.330	0.1834
Semirural - Rural	*C. vicina*	-0.0142	0.1840	-0.077	0.93841
Semirural - Rural	*C. vomitoria*	-0.3263	0.2134	-1.529	0.126

2.3.4 Effects of habitat

The factor 'habitat' had a significant effect on the fly catch for all the species collected (Table 2.4). The calliphorid community in 'open' habitats was dominated by *L. sericata* (Fig. 2.7). The rare species, *L. richardsi*, was also more frequently found in this habitat it was rarely found in hedgerows and it was not found at all in woodland traps (Fig. 2.7). There was no significant difference in the number of *L. richardsi* caught in hedgerow or open habitats (Table 2.4).

The most abundant species in hedgerow habitats were *L. caesar* (Fig. 2.7) and *C. vicina*. Statistical analysis showed that the abundance of the latter species was not significantly different between woodland and hedgerow habitats ($z=-0.172$, $P=0.86$, Table 2.4). Although this species was found in shaded habitats, it was also found in open habitats in smaller

numbers (Fig. 2.7). In contrast, *L. ampullacea* had its highest abundance in woodland habitats and was almost completely absent from 'open' environments (Fig. 2.7).

Woodland habitats were dominated by *L. caesar* and *C. vomitoria* (Fig. 2.7). The abundance of *L. caesar* was significantly different between habitat types (Table 2.4), with its highest abundance recorded in woodland and its lowest in 'open' habitats (Fig. 2.7). Unlike *C. vicina*, statistical analysis showed a significant difference between hedgerow and woodland habitats on the abundance of *C. vomitoria* (Table 2.4), which had higher abundancies in the latter habitat (Fig. 2.7, Fig. 2.13).

2.3.5 Interaction between factors

There was a significant interaction between habitat and site on the catch of *L. sericata, L. caesar*, *C. vicina* and *C. vomitoria* (Table 2.2). The sheep blowfly, *L. sericata,* was more common in the hedgerow habitats of the semirural farm (Fig. 2.8) than those of the rural farm ($z=-7.142$, $P<0.001$). Similarly, although *L. caesar* was more abundant in shaded habitats, it was not restricted to 'open' habitats, this happened with more frequency in the semirural farm than it did in the rural farm ($z=-4.508$, $P<0.001$; Fig. 2.9). The bluebottle, *C. vicina,* which also was generally more abundant in shaded habitats, was recovered more frequently from the 'open' habitats of the rural farm than those of the semirural farm ($z=4.22$, $P<0.001$; Fig. 2.12).

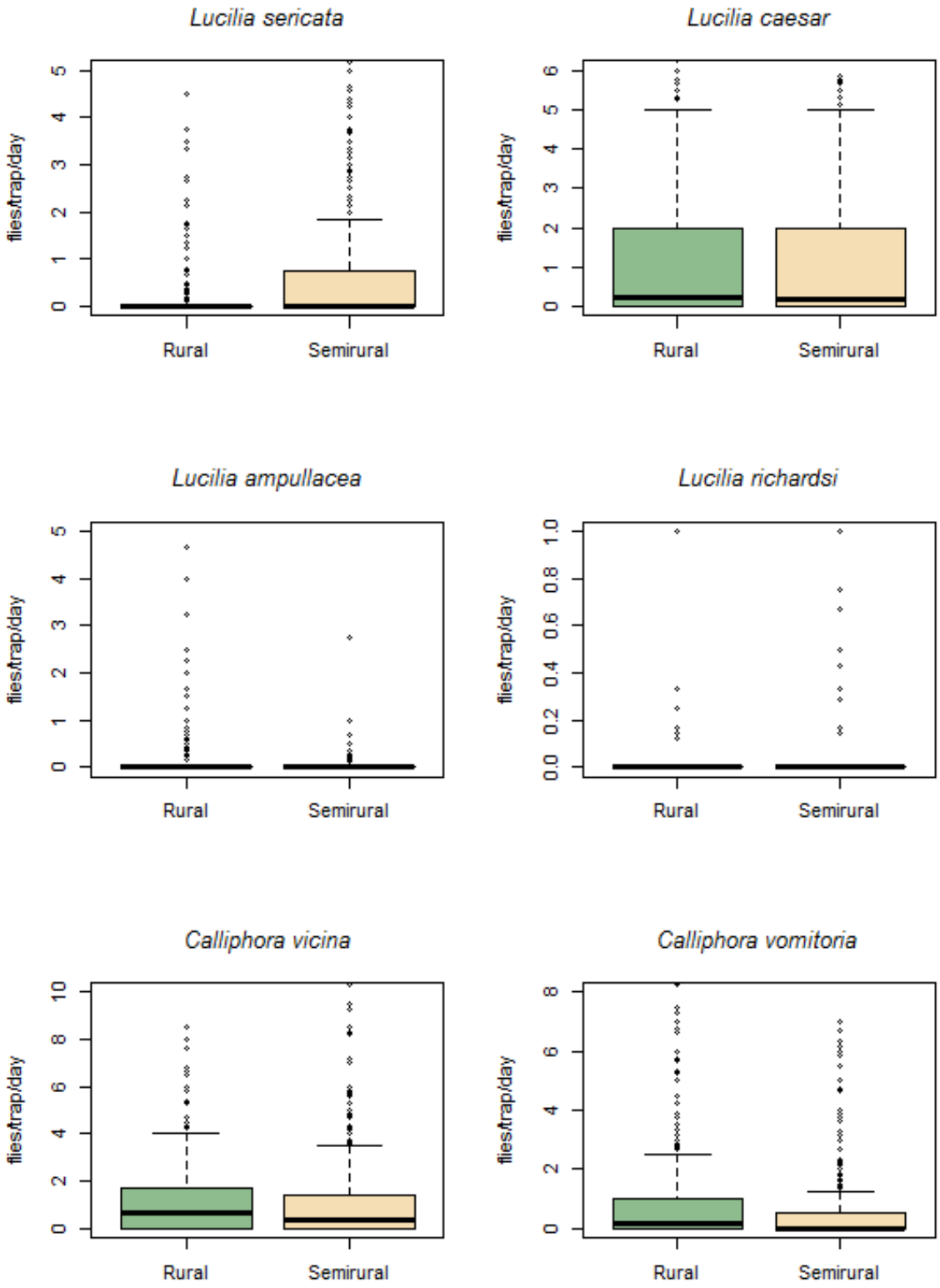

Figure. 2.6. The number of flies caught (trap/day) at rural or semirural sites. horizontal axis displays the site of study (rural and semirural). The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots). Individual species names are indicated at the top of their respective figure.

Table 2.4. Effects of habitat on fly abundance. Table displays the estimate, standard error, z value and p values computed for individual calliphorid species studied.

Sp.	Factor	Estimate	SE	z	Pr(>|z|)
L. sericata	open – hedge	0.75761	0.28256	2.681	0.007336
	woods - open	-4.13423	0.44628	-9.264	<2e-16
	woods - hedge	-3.37662	0.45041	-7.497	6.54E-14
L. caesar	open – hedge	-2.49063	0.25639	-9.714	<2e-16
	woods - open	2.97109	0.25492	11.655	<2e-16
	woods - hedge	0.48045	0.23309	2.061	0.0393
L. ampullacea	open – hedge	-3.47E+01	3.42E+06	0	0.999992
	woods - open	3.737E+01	3.419e+06	0	0.999991
	woods - hedge	2.67E+00	3.10E-01	8.601	< 2e-16
L. richardsi	open – hedge	2.46E+00	5.99E-01	4.107	4.01E-05
	woods - open	-3.56E+01	3.881e+06	0	0.99999
	woods - hedge	-3.32E+01	3.88E+06	0	0.99999
C. vicina	open – hedge	-1.1946	0.18844	-6.339	2.31E-10
	woods -open	1.1627	0.1885	6.169	6.88E-10
	woods - hedge	-0.03191	0.18572	-0.172	0.86359
C. vomitoria	open – hedge	-2.87744	0.26565	-10.832	< 2e-16
	woods - open	4.43343	0.26322	16.843	< 2e-16
	woods - hedge	1.55598	0.21063	7.387	1.50E-13

2.4 Discussion

Carrion is an unpredictable and ephemeral resource and diversity within carrion breeding insect community is thought to be structured by intense resource competition with ecophysiological, behavioural or phenological differences allowing niche partitioning in space and time (Cruickshank and Wall, 2002). However, the interspecific ecological difference that facilitate coexistence within the blowfly community are not fully understood. Differences in habitat use may have an important impact on their population dynamics, through its effects on the intensity of competition, predation or parasitism (Hatcher et al., 2006). Understanding patterns of habitat use is also important, because heterogeneity in distribution affects the variance in catch and thereby determines the spatial scale, method and intensity at which sampling must be carried out (Southwood, 1976). For insect pests, the pattern of habitat use and the spatial scale of aggregation with specific parts of the habitat has a critical influence on the efficacy of almost all control techniques and therefore on the nature, application practicalities and cost of any control procedure. An understanding of the factors that determine the relative abundance of *Lucilia* is also of particular practical interest since *L. sericata* and *L. caesar* are of economic importance in livestock myiasis (MacLeod, 1943; Wall et al., 1992a).

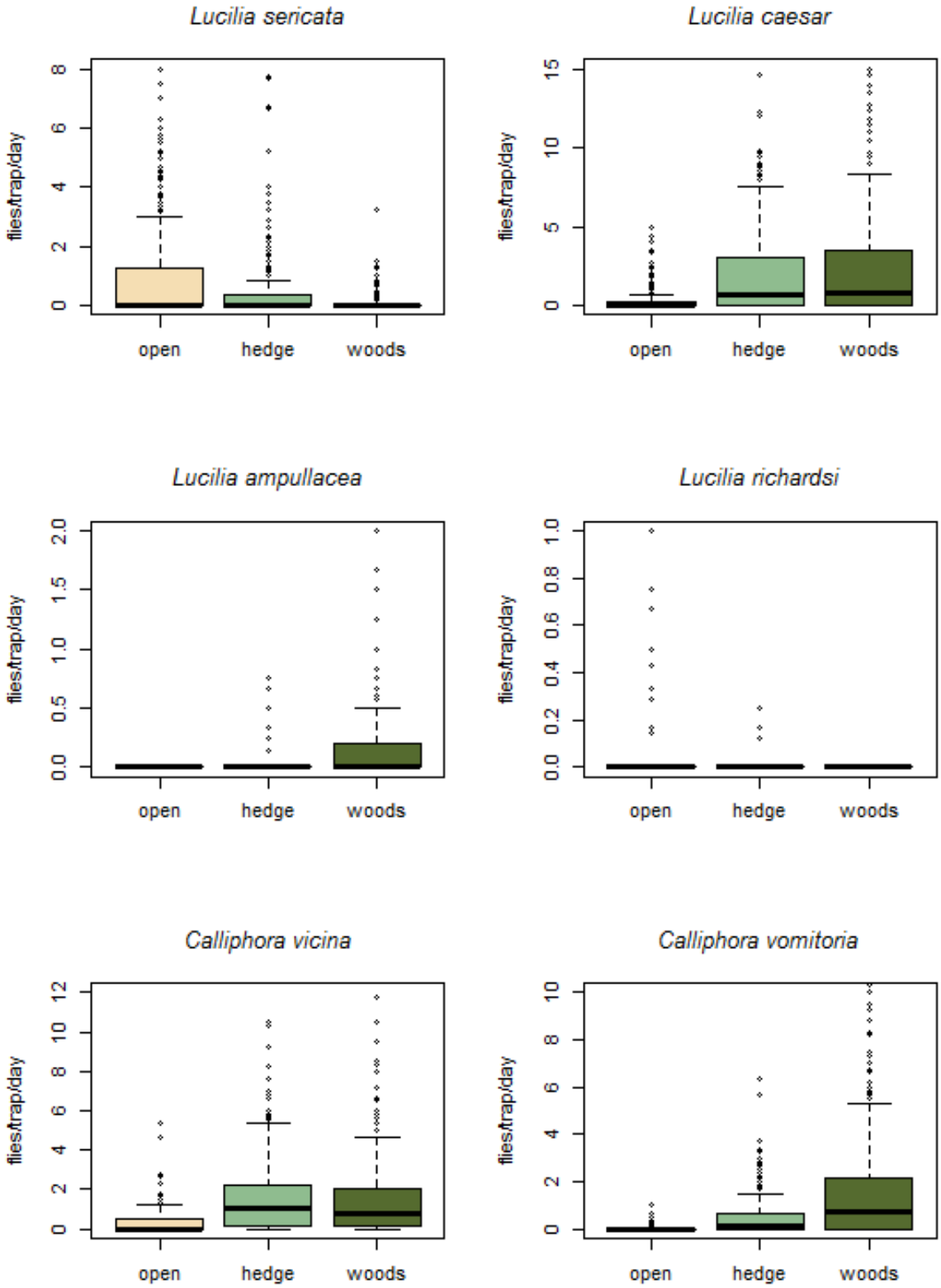

Figure. 2.7 The number of flies caught (trap/day) in open (cream), hedgerow (light green) and woodland (dark green) habitats. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots). Species names are indicated at the top of their respective figure.

Ecological studies typically try to explain distribution patterns of organisms and their variability through space and time. This is often made by counting individuals in a certain range (Southwood, 1976). Different statistical approaches have been employed by field entomologists to model general patterns of insect species distribution evaluating the effects of a series of variables (Southwood, 1976). Although there is a relatively wide body of research on blowfly ecology, spatial and/or temporal distribution of calliphorid species of veterinary/forensic importance is typically modelled assuming homogeneity of variance and normal distribution of the data. However, datasets often exhibit a high proportion of zeros, where the data is skewed by a mean that exceeds the theoretical variance. This phenomenon is called overdispersion and it is rarely considered in statistical analysis on insect ecology (Sileshi, 2006). If the model is not properly selected, the presence of excess zeros and variance heterogeneity can invalidate its assumptions. Furthermore, it could lead to a biased estimation on the effects of ecological factors that are defining the variability of a pattern. Relatively recent research has shown that negative binomial distribution and over-dispersed corrected Poisson models can provide better estimates on the probability distribution of insect datasets that display overdispersion (Sileshi, 2006). In order to provide better estimates on the effect of ecological on spatial and temporal distribution of saprophagous and facultative blowflies, this work employed a generalised linear model with negative binomial distribution for data analysis. This work aims to provide an accurate inference of the effects of habitat, temperature and farm type on the spatial and temporal abundance of a saprophagous/facultative calliphorid community.

The present work took place over a single year and, given the highly variable weather of the UK, climatic variation between years might be expected to result in differences in the abundance of the various species recorded. More extensive research over several years would be required to assess this possibility. However, the findings presented here correspond with previous studies on spatial and temporal distribution of calliphorid flies, giving confidence in the underlying robustness of the trends identified. For example, studies have shown that C*alliphora* species to be more abundant in cooler months relative to *Lucilia* species (Greco et al., 2014; Zabala et al., 2014). The minimum temperature below which the development of *C. vicina* ceases was reported to be 2 °C (Greenberg, 1991) and recent laboratory studies estimated a minimum developmental temperature of 1°C and a requirement of 4,700 accumulated degree hours for the development from the egg hatch to pupation (Donovan

et al., 2006). The reduction in the abundance of *Calliphora* species in the carrion community during the warmer months could also possibly be affected also by the presence of other carrion breeding species with higher threshold temperature development, increasing competition for food resources (Fig. 2.5). The lower threshold temperature for *L. sericata* development is 9 °C (Wall et al., 1992b) and mean temperatures above 9 °C were not achieved until late April in the year of the study. Hence, *Lucilia* adults were not present during March and most of April (Figs. 2.8-2.11). When *L. sericata* specimens were present, they were significantly more abundant in open habitats and rarely seen in the woodland (Fig. 2.8). The high abundance of *L. sericata* in open relative to other habitats has also been recorded previously (Gregor, 1991; Smith and Wall, 1997b; Martínez-Sánchez et al., 2001). This pattern of habitat use may be related to light intensity and previous studies have suggested that the behaviour (Smith et al., 2002) and trap catch size (Wooldridge et al., 2007) of *L. sericata* are strongly affected by light intensity, although microclimatic temperature and humidity tolerances may also be important(Cruickshank and Wall, 2002). The data also support previous studies where it is suggested that *L. sericata* is a synanthropic species(Fischer, 2000; Hwang and Turner, 2006), as the number of specimens recorded for the rural farm was much lower than that one recorded for the semirural farm, and in fact, during the whole experiment this number did not surpass the 0.8 flies/trap/day in the rural farm.

Lucilia sericata

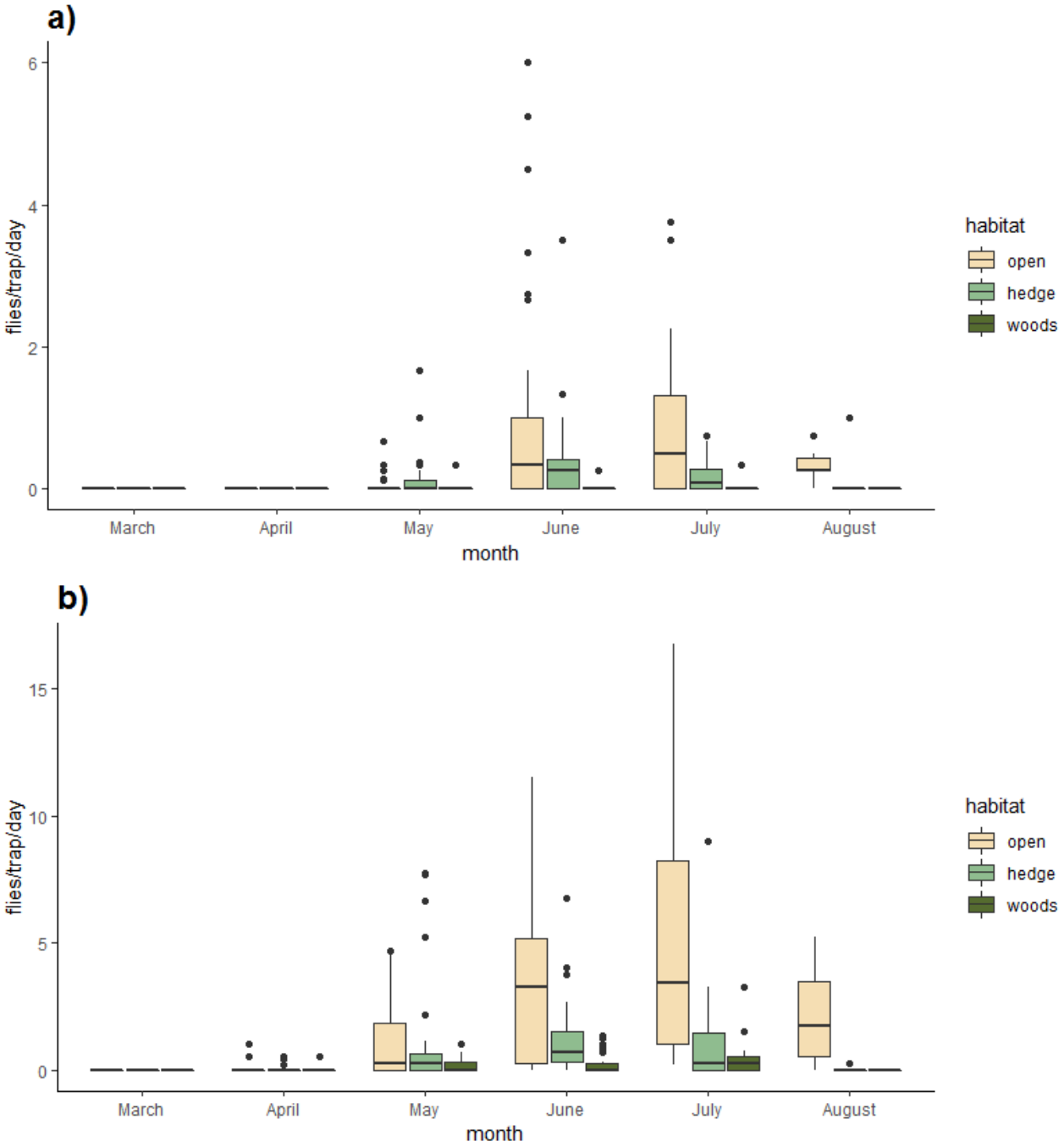

Figure. 2.8. The number of *Lucilia sericata* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

Lucilia caesar

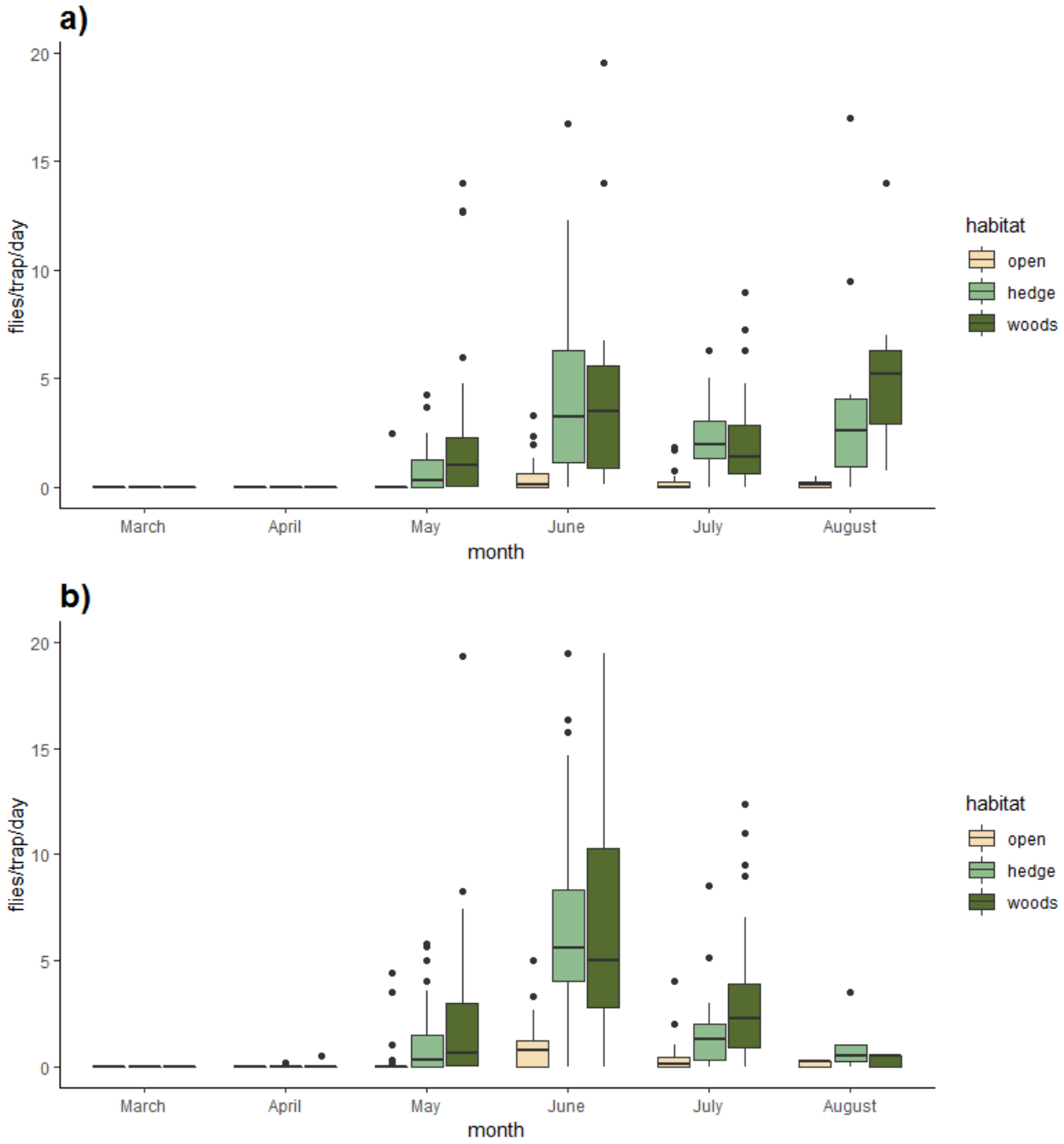

Figure 2.9. The number of *Lucilia caesar* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

Lucilia ampullacea

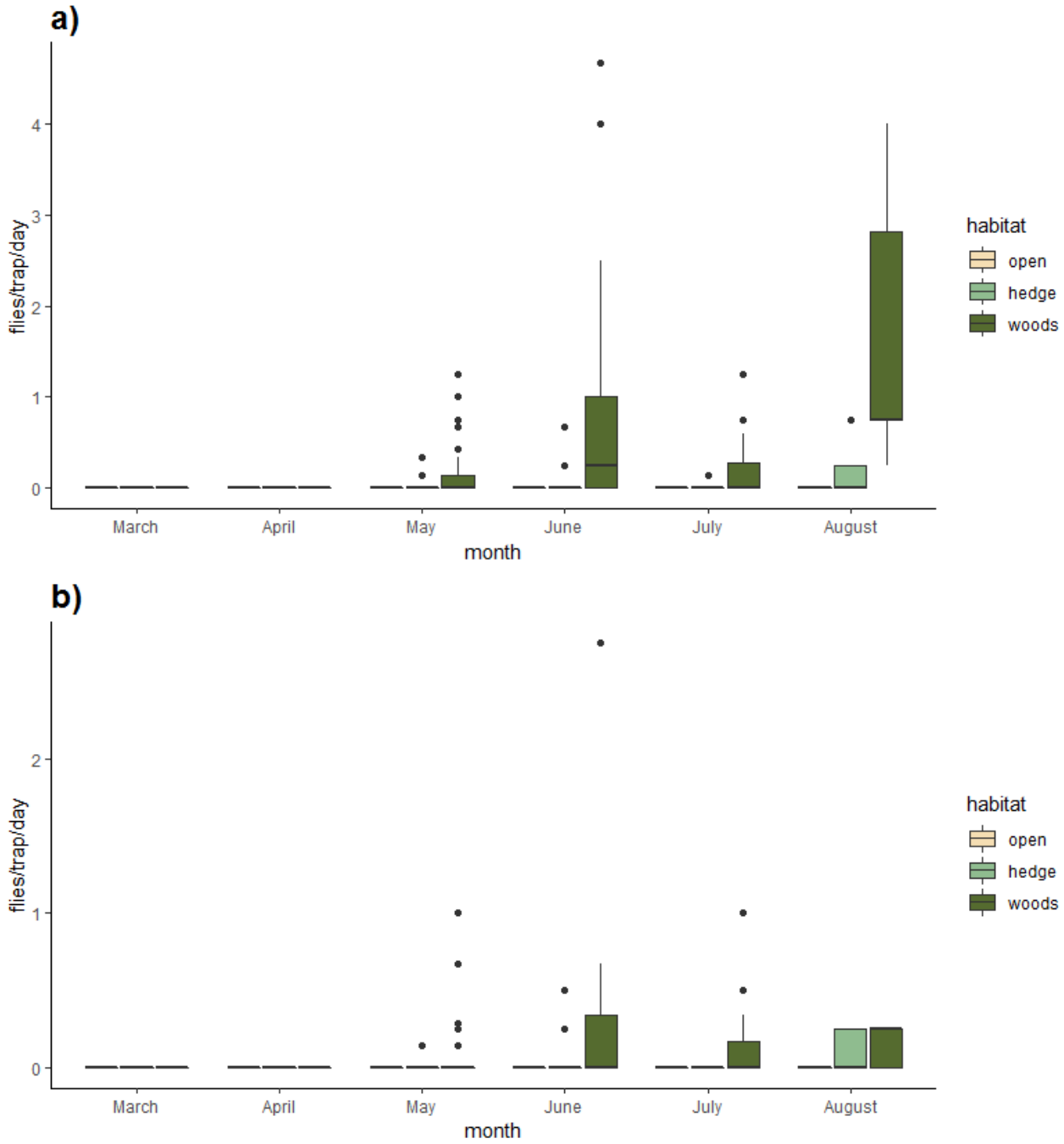

Figure 2.10. The number of *Lucilia ampullacea* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

Lucilia richardsi

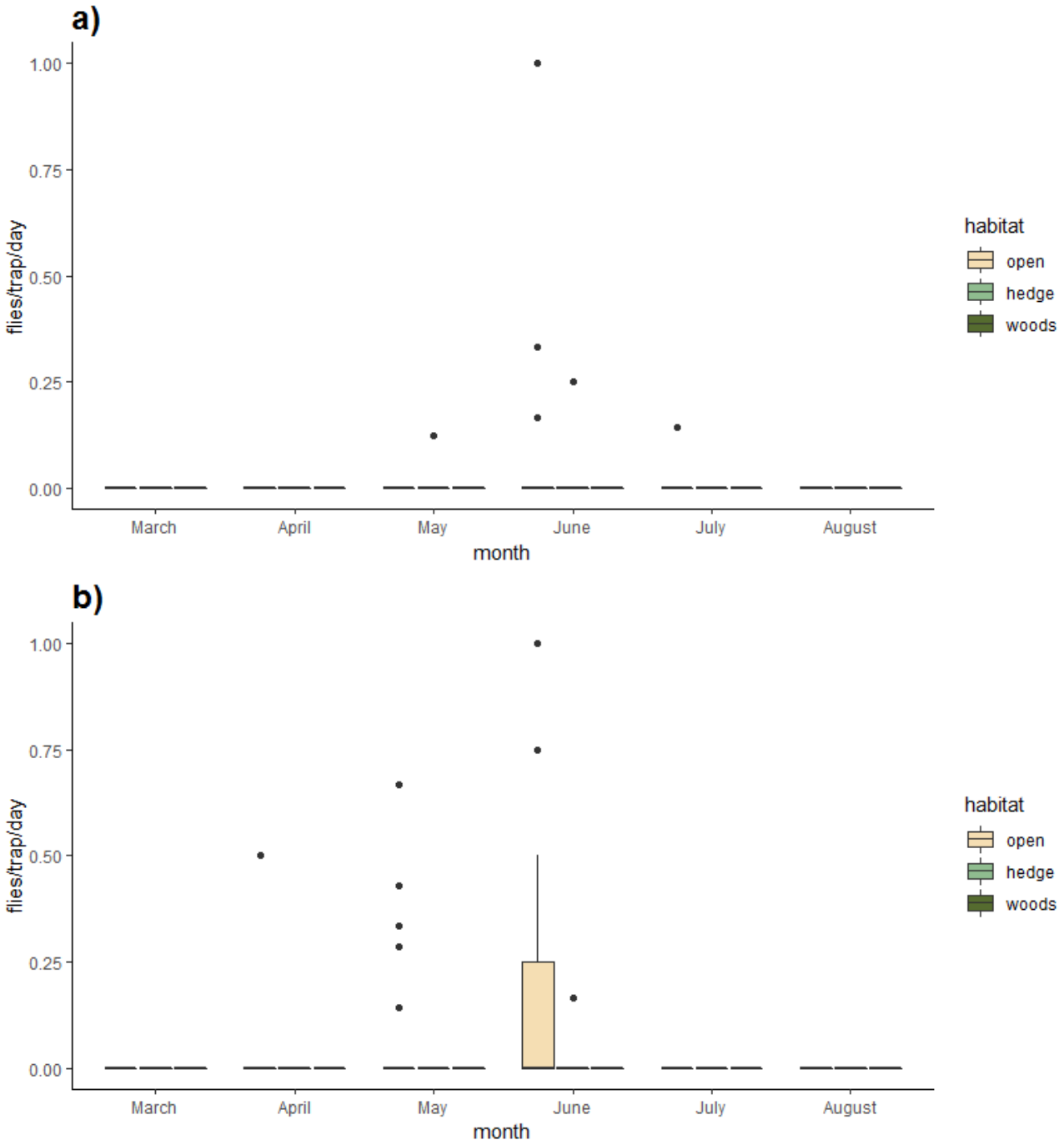

Figure 2.11. The number of *Lucilia richardsi* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

Calliphora vicina

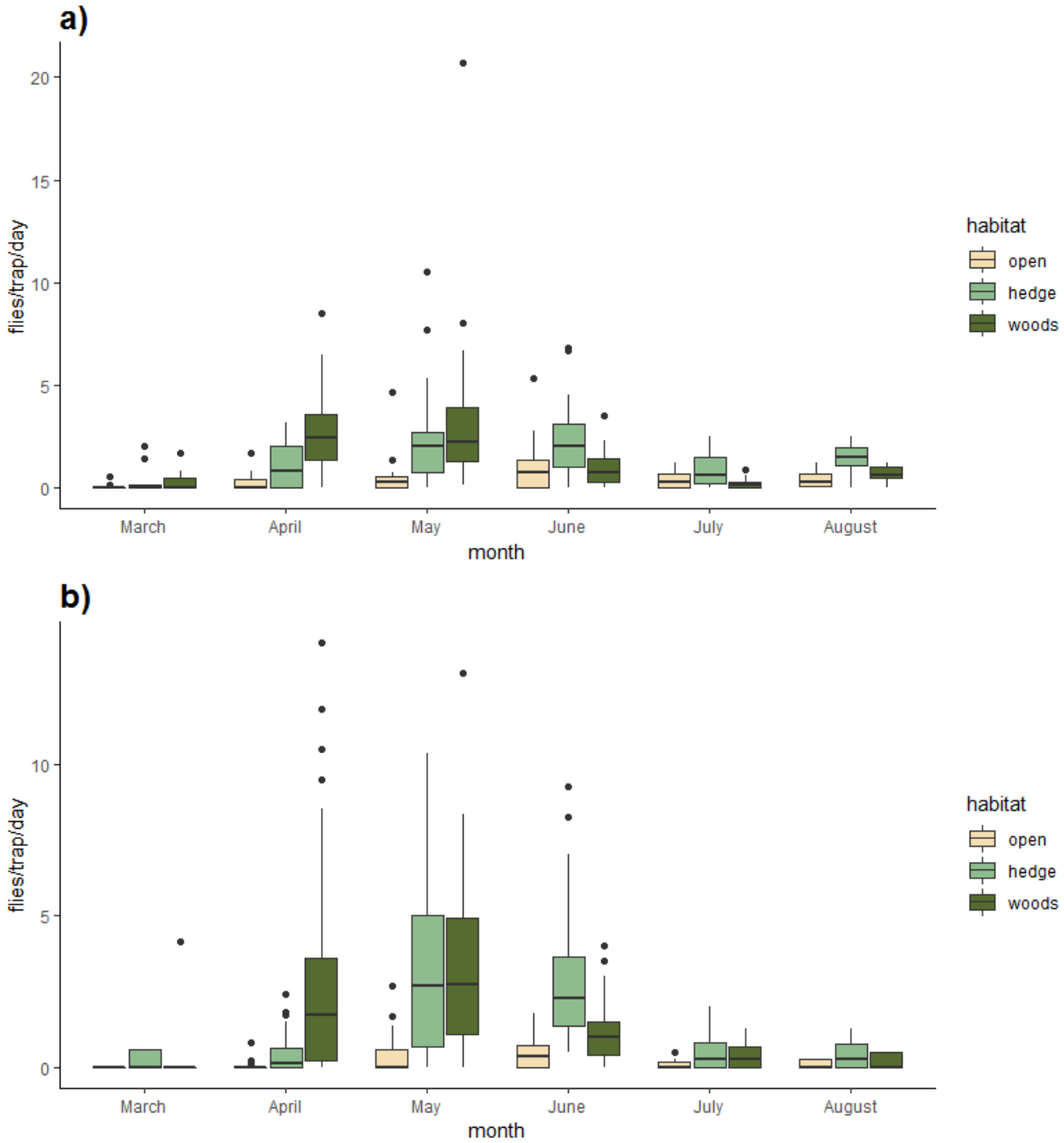

Figure 2.12. The number of *Calliphora vicina* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

Calliphora vomitoria

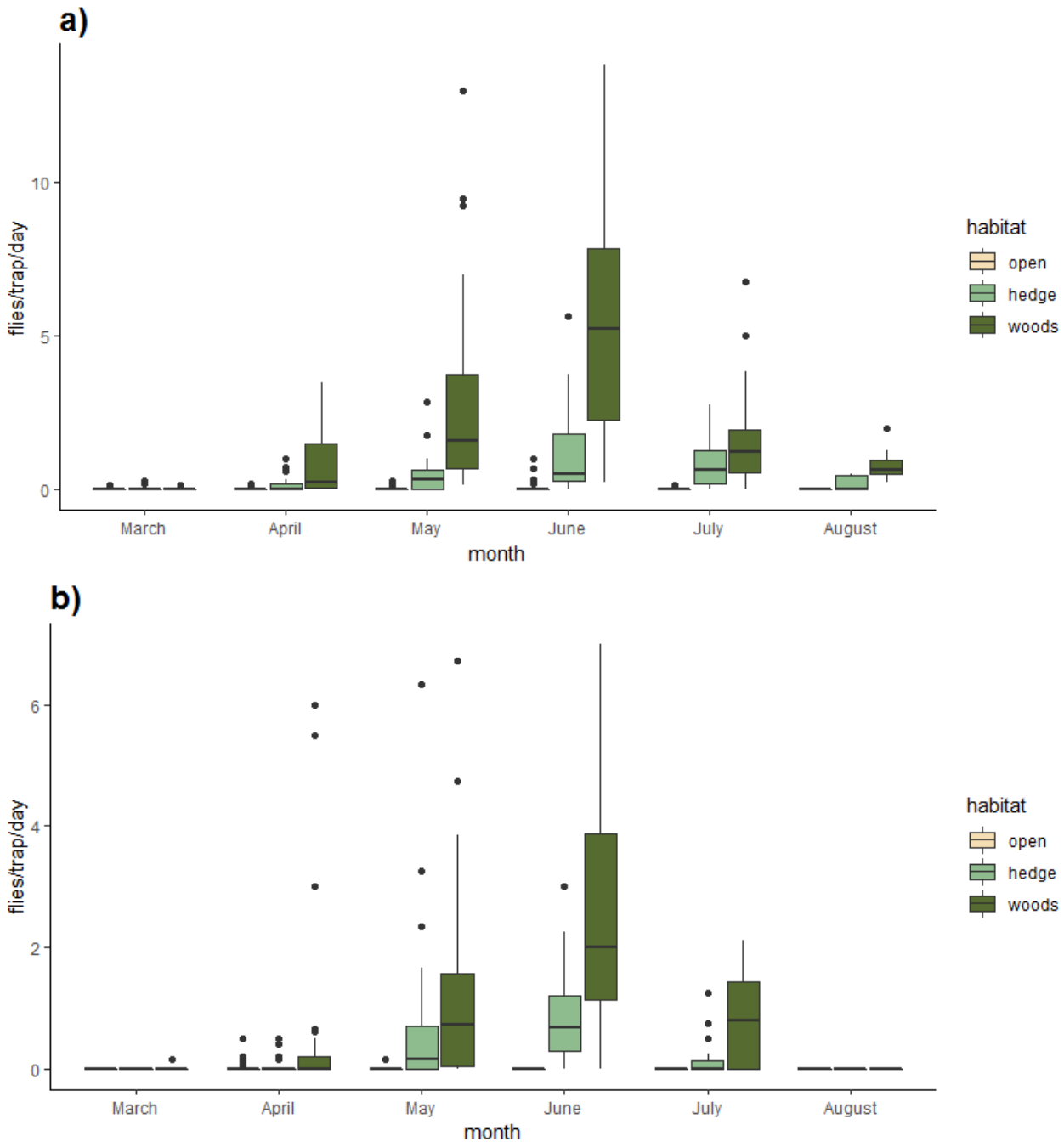

Figure 2.13. The number of *Calliphora vomitoria* caught (trap/day) in different months and in different habitats (open, hedge and woods) at different sites: a) rural; b) semirural. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots)

A species that is almost identical to *L. sericata* in their morphology, *L. richardsi*, was also more frequently found in the same habitats, usually 'open' (Fig. 2.11), which suggest also a close affinity in their physiology or behaviour. It is also known that *L. richardsi* has a close phylogenetic relationship with *L. sericata* (McDonagh and Stevens, 2011*)*. Despite their similarity and close relationship, it remains unclear why *L. richardsi* has never been reported involved in sheep strike.

In the present study, *L. caesar* was the most abundant calliphorid species, collected mainly from shaded habitats (Fig. 2.9). In western Scotland *L. caesar* was the second most abundant species of myiasis agent in sheep strikes and occurred at a significantly higher frequency than in more southerly latitudes (Morris and Titchener, 1997). While it has been suggested that this may be due to lower temperature and higher humidity requirements for *L. caesar* (Wall et al., 1992a) there is no firm understanding of why the involvement of *L. caesar* in ovine cutaneous myiasis is rare in England although it is very common as a carrion breeding species in woodland habitats. Similarly, *L. illustris* is reported as a common species involved in flystrike cases in Norway (Brinkmann, 1976) and as the dominant species of the carrion fly community in Finland (Hanski and Kuusela, 1977). However in England *L. illustris* has not been reported involved in flystrike (Wall et al., 1992a) and**,** in fact, the data presented here alongside previous studies suggest that it is a rare species in England (Smith and Wall, 1997b). Nonetheless, it is known that, unlike *L. caesar*, it can be found in most habitat types (MacLeod and Donnelly, 1956). The reason why *L. illustris* is rarely involved in flystrike cases in the UK is still unknown and further work is required to study this phenomenon. Another species of the *L. caesar* species group, *L. ampullacea*, exhibited stronger affinity to shaded habitats, as there were no specimens recovered from open habitats at all (Fig. 2.10). It is likely that this species is confined to locations with higher humidity levels and low light intensities.

Most natural carcasses are situated in shaded habitats, as dying animals seek shelter (Blackith and Blackith, 1990) and this means that the food resource available to *L. sericata* is limited, as it has been reported as a relatively poor inter-specific competitor with other calliphorid species (Smith and Wall, 1997a). This could have played an important role in niche partitioning, possibly forcing *L. sericata* to migrate to food resources with fewer potential competitors, such as living hosts in open habitats. However, more studies need to be carried out to determine and understand the pathway of the evolution of parasitic behaviour within this genus.

MacLeod and Donnelly (1956) suggested that relatively persistent fly distributions within the vegetational mosaic might be delimited by habitat requirements. Regardless of the long interval time between the present study and the former, the present results match largely and support the findings of the former study. The data presented here suggest that differences in phenology and habitat use between *Calliphora* and *Lucilia* are likely to be mediated most strongly by differences in temperature tolerance, whereas difference within species of the genus *Lucilia* are likely to be mediated by differences in humidity and desiccation tolerance and light intensity, resulting in effective niche partitioning. Desiccation tolerance may have conferred a behavioural advantage for *L. sericata* over other blowfly species, allowing it to become a more common agent of livestock myiasis in open pasture. Nonetheless, more studies are required to confirm this.

3. Species composition in amphibian myiasis in Europe and taxonomic status of the toad fly *Lucilia bufonivora*

Contributions

Gerardo Arias-Robledo was the main researcher and undertook DNA extractions, PCR procedures, sequence assembling, editing, statistical analysis and wrote the drafts of the manuscript. Jamie Stevens assisted as the main supervisor and supported with manuscript corrections and interpretation of results. Richard Wall supported with general advice and corrections on the manuscript. Andy King assisted with a PCR reaction and advice on molecular procedures. Tom Jenkins offered guidance on the DNA extraction protocols. T Stark, G. Guex, S. Henderson, L. Griffiths, A. Breed, B. Lawson, J. Groen, C. Laurijssen, R. Koelman, J Mosteart, D. Mebs and F. Arias provided samples for analysis. The Molecular Ecology and Evolution research group provided lab consumables (University of Exeter). The work was funded by CONACyT and it was published in the journal **Medical and Veterinary Entomology** (see **appendix I**).

3.1 Introduction

Within the genus *Lucilia*, only one species is thought to behave as an obligate parasite in Europe: *Lucilia bufonivora* (Moniez) (Brumpt, 1934). Moniez (1876) described the fly after he succeeded in rearing twenty-five adults which were then designated as syntypes in Raismes (Nord), France, however with no specified number of males and females. This species has a high host-specificity for amphibians and is thought to be the main amphibian myiasis agent in the Palearctic (Strijbosch, 1980; Weddeling and Kordges, 2008; Martín et al., 2012). Eggs are laid on the living host and, after hatching, the first stage larvae migrate to the nasal cavities where larval development takes place (Fig. 3.1a-b), usually resulting in the death of the amphibian host (Brumpt, 1934; Zumpt, 1965). *L. bufonivora* has been reported as the cause of myiasis in a range of amphibian hosts, however, most reports relate to infestations of the common toad, *Bufo bufo* (Strijbosch, 1980; Weddeling and Kordges, 2008; Martín et al., 2012). This blowfly is widely distributed in Europe (Rognes, 1991) and Asia (Fan et al., 1997). Although it was thought to be restricted to the Nearctic, adult samples were recently reported from Canada (Tantawi and Whitworth, 2014).

One of the main limitations of Dipterology, in general, is the close morphological resemblance between closely related species. This phenomenon has led to misidentification and erroneous reports of biodiversity data (Rognes, 2014). Certainly, *L. bufonivora* is almost morphologically identical to a saprophagous species *Lucilia silvarum* (Rognes, 1991; Tantawi and Whitworth, 2014). According to Rognes (1991), the *L. bufonivora* group is characterised by the presence of a normal arista with long hairs; 2-4 median marginal setae on T3 strong, different from the paramedian setae, and as long as T4 (or longer than half the length); the first flagellomere half as long as the greatest length of eye viewed in profile or less. Differentiation between *L. bufonivora* and *L. silvarum* is normally carried out by the number of *post acr* bristles, two and three respectively (Aubertin, 1933). However, this morphological character is very variable (Rognes, 1981, 1991), and thus accurate identification should include thorough examination of the terminalia as indicated by Rognes (1991) and Tantawi and Whitworth (2014).

L. silvarum is widely distributed blowfly species in the Palearctic (Rognes, 1991) and the Nearctic (Hall, 1948). In Europe, however there are several reports of *L. silvarum* being involved in amphibian myiasis (Duncker, 1891; Mortensen, 1892; Linder, 1924; Stadler, 1930). It is also known to behave mainly as a carrion breeder in this region (Zumpt, 1956;

Fremdt et al., 2012). Zumpt (1965) argued that, in the Palearctic, most records of toad myiasis thought to have been caused by *L. silvarum* might be due to misidentification and should be attributed to *L. bufonivora*. To date, species composition in amphibian myiasis in Europe remains unclear due to taxonomic confusion. Moreover, no molecular studies have yet confirmed the involvement of either species in amphibian myiasis in Europe

The monophyly of *Lucilia* as a genus has been debated for decades. Indeed, Rognes (1991) argued that, at the time he was writing, no detailed phylogenetic analyses had been performed, and thus the monophyly of *Lucilia* with respect to *Hemipyrellia* and *Hypopygiopsis*, remained questionable since they differed only in the absence of a long fine setae on the katerguite (Zumpt, 1965). Using a multi-gene approach, Williams et al. (2016) noted that recognising *Hemipyrellia* as a genus would consistently leave *Lucilia* paraphyletic.

On the American continent, Townsend (1919) proposed a new genus, *Bufolucilia,* which included *L. bufonivora* as the type species, along with *L. silvarum*. Subsequently, Hall (1948) included *Lucilia elongata* Shannon in this genus, which has also been reported as an amphibian parasite in North America (James and Maslin, 1947; Bolek and Janovy, 2004). This has created confusion on the taxonomic status of *L. bufonivora*. More recently, the genus *Bufolucilia* was dismissed as a synonym of *Lucilia* by Rognes (1991), although it continues to be used as a subgenus by a number of authors (Kraus, 2007; Verves and Khrokalo, 2010; Draber-Mońko, 2013). However, while several studies provide strong support for the grouping of *L. bufonivora* and *L. silvarum* as closely related sister species (Stevens and Wall, 1996; McDonagh and Stevens, 2011) recognition of subgenus *Bufolucilia* would leave other *Lucilia* species in a heterogeneous and paraphyletic group, as observed with some other proposed (but poorly supported) genera, for example, *Phaenicia* (Stevens and Wall, 1996).

The mitochondrial gene cytochrome oxidase subunit one (*COX1*) has proved to be a useful molecular marker for detection and identification of parasites and pathogens such as nematodes (Aravindan et al., 2017), trypanosomes (Rodrigues et al., 2017), ticks (Chitimia et al., 2010), oestrid flies (Samuelsson et al., 2013) just to mention a few. Additionally, it has been widely used for blowfly phylogenetics (McDonagh and Stevens, 2011; Williams and Villet, 2013; Williams et al., 2016). Nonetheless it has its own limitations, for example in some cases molecular diagnostics of closely related species are not entirely reliable (Nelson et al., 2007; Whitworth et al., 2007). To overcome this problem, previous studies have

employed multi-gene approaches that not only improve identification accuracy, but also phylogenetic resolution at different levels of divergence (Wallman et al., 2005).These studies have made use of not only just mtDNA sequence data, but also ribosomal non-coding DNA and nuclear DNA (McDonagh and Stevens, 2011; Williams and Villet, 2013; Williams et al., 2016; Yusseff-Vanegas and Agnarsson, 2017).

The aim of this work was to examine the blowfly species composition in amphibian myiasis in Europe by extracting and analysing DNA of unidentified larval specimens that were found causing nasal-myiasis in live-hosts. Molecular analysis consisted of a multi-gene approach using sequence data from the mitochondrial protein-coding gene cytochrome c oxidase subunit I (*COX1*) and the nuclear gene elongation factor 1 alpha (*EF1a*). It also aimed to resolve the taxonomic confusion of the proposed genus '*Bufolucilia*' (Townsend, 1919) and the positioning of *L. bufonivora* and *L. silvarum* as distinct species.

3.2 Materials and methods

3.2.1 Adult and larval specimens

Unidentified larval specimens were sampled from 16 separate toad myiasis cases from six different locations in Britain (8 cases), four locations in The Netherlands (7 cases) and one site in Switzerland (1 case) (Table 3.1). Four adult specimens of *L. bufonivora* were also analysed, two from southern Germany and two collected with the aid of baited traps in The Netherlands (Table 3.3, Fig. 3.2). Five adult specimens of *L. silvarum* were analysed, including three from the UK, one from the USA and one from The Netherlands. A specimen of *L. elongata* from Alberta, Canada was also added to facilitate further exploration of the evolutionary relationships across the broader group of fly species reported as amphibian parasites. Additionally, adult specimens of *L. sericata, Lucilia caesar*, *Lucilia richardsi* and *Lucilia ampullacea* collected in Bristol, UK (as described in chapter 2) were added to the analysis (Table 3.3). Two adult specimens of toad fly *L. bufonivora* and one adult specimen of *L. silvarum* collected in the Netherlands (as will be described in chapter 5) were also added to the analysis (Table 3.3).

Information on the evolutionary relationships between Palearctic and strictly Nearctic lineages of *Lucilia* (e.g. *Lucilia mexicana* Macquart) is very limited. Given that *L. elongata* is strictly Nearctic (Tantawi and Whitworth, 2014) the addition of a non-parasitic taxon

restricted to this area might help resolving whether *L. elongata* has close relationships with other Nearctic species of non-parasitic blowflies. Therefore, two new specimens of adult *Lucilia mexicana* from Chapingo, Mexico were included in the analysis (Table 3.3).

Figure 3.1. Common toad (*Bufo bufo*) with nasal myiasis due to *Lucilia bufonivora*. a) early stage of the disease, small nasal myiasis wounds are visible at each nostril. b) advance stages of nasal myiasis posterior ends of live third instar larvae are visible within the enlarged wounds. Bridgnorth, Shropshire, U.K.; Photographs courtesy of Dr. A. Breed, Animal and Plant Health Agency, Defra, U.K

Sequence data for specimens *Lucilia cuprina* and *Lucilia illustris* were obtained from EMBL/GenBank and included in the analysis. Two adult samples of *Calliphora vicina* collected in the UK (as described in chapter 2) and were included as outgroup taxa. Finally, a laboratory-reared adult specimen sent from Switzerland and labelled '*L. bufonivora*' was included in the analysis. This specimen belonged to a second generation of flies from a colony that was originally stablished by rearing larvae from a toad-myiasis case in Switzerland (Table 3.3). All specimens were stored in 100% ethanol at 4°C prior to analysis.

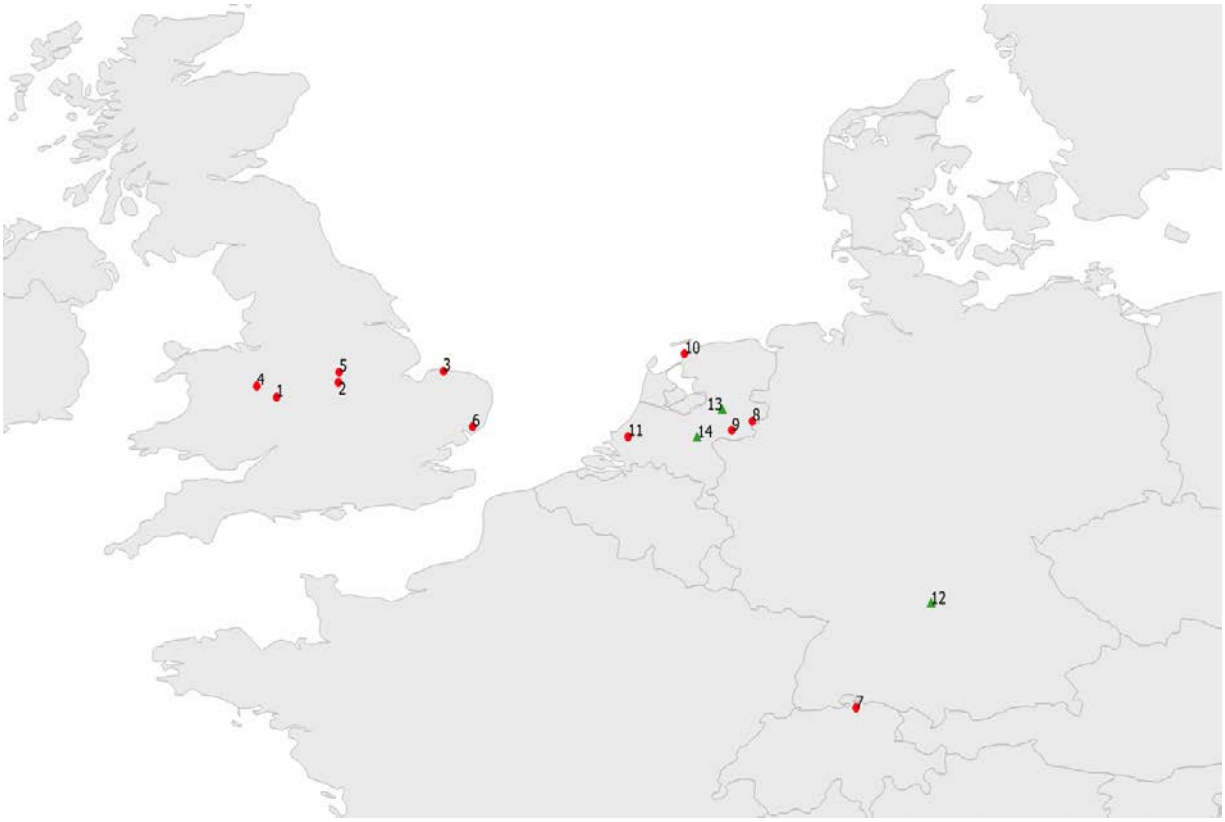

Figure 3.2. Location of larval (red dots) and adult (green triangles) specimens of *Lucilia bufonivora* analysed in this study. 1=Bridgnorth (3 cases); 2=Loughborough (1 case); 3=Holkham (1 case); 4=Shrewsbury (1 case); 5=Nottingham (1 case); 6=Suffolk (McDonagh and Stevens, 2011); 7=Ossingen, CHE (1 case); 8=Haasksbergen, NLD (4 cases); 9=Zelhem, NLD (1 case); 10=Friesland, NLD (1 case); 11=Rotterdam, NLD (1 case); 12=Baden-Württemberg, DEU (2 adult flies); 13=Olst, NLD (1 adult); 14=Winssen, NLD(one adult).

3.2.2 DNA extractions and Polymerase Chain Reaction procedures

Thoracic muscle of adult specimens was used for DNA extraction to avoid contamination with ingested protein, eggs or parasites. To avoid potential contamination from larval gut contents, the anterior and posterior ends of larvae were used for DNA extraction from LII and LIII life stages, while whole specimens were used if samples were LI; live larvae were maintained on damp filter paper for 3–6 hours prior to storage in ethanol to allow them to evacuate their gut contents. DNA extractions were carried out using a QIAGEN DNeasy® Blood and Tissue Kit (Qiagen GmbH, Germany) according to manufacturer's instructions.

Table 3.1. Larval *Lucilia* specimens studied, including the location of collection, infestation ID and accession numbers for EMBL/GenBank DNA sequences for both *COX1* and *EF1a*.

Infestation ID	Larvae analysed	Country/Region of origin	Name on tree	*COX1*	*EF1α*
BB016-2	1	Haaksbergen, The Netherlands	Lbufo(NLD1)	FR719161	FR719238
BB016-3	1	Haaksbergen, The Netherlands	Lbufo(NLD2)	FR719161	FR719238
BB016-1	1	Zelhem, The Netherlands	Lbufo(NLD3)	FR719161	FR719238
BB016-4	1	Haaksbergen, The Netherlands	Lbufo(NLD4)	FR719161	FR719238
BBSP1	1	Haaksbergen, The Netherlands	Lbufo(NLD5)	FR719161	FR719238
Friesl-1	1	Friesland, The Netherlands	Lbufo(NLD6)	FR719161	FR719238
Rott-1	1	Rotterdam, The Netherlands	Lbufo(NLD7)	FR719161	FR719238
Oss-Ch-1	1	Ossingen, Switzerland	Lbufo(CHE)	FR719161	FR719238
WV15 6QR-1	1	Bridgnorth, Shropshire, UK	Lbufo(GBR1)	FR719161	FR719238
WV15 6QR-2	1	Bridgnorth, Shropshire, UK	Lbufo(GBR2)	FR719161	FR719238
XT767-16	1	Loughborough, UK	Lbufo(GBR3)	FR719161	FR719238
XT931-16	1	Bridgnorth, Shropshire, UK	Lbufo(GBR4)	FR719161	FR719238
Holk-1	2	Holkam, UK	Lbufo(GBR5), Lbufo(GBR6)	FR719161 FR719161	FR719238 FR719238
Shrew-446	2	Shrewsbury, UK	Lbufo17, Lbufo(GBR8)	FR719161 FR719161	+LT900481 FR719238
ott-1	2	Nottingham, UK	Lbufo(GBR9) Lbufo(GBR10)	FR719161 FR719161	FR719238 FR719238
Suff-1	2	Suffolk, UK	Lbufo(Suff1) Lbufo(Suff2)	FR719161 FR719161	FR719238 FR719238

+ = new sequence. NOTE: Only new sequence data were submitted to GenBank as haplotypes, thus specimens with the same haplotype were allocated with the same accession codes.

DNA was extracted as total nucleic acid and subjected to PCR to amplify the cytochrome oxidase I (*COX1*) region of the mitochondrial protein-coding gene and the *EF1-EF4* region of the nuclear protein-coding gene elongation factor 1 alpha (*EF1a*). Universal insect primers previously published (Table 3.2) were used. The PCR protocol published by Folmer et al. (1994) was modified to amplify *COX1* and *EF1-EF4* with the following cycling conditions: 94°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C (*COX1*) or 48°C (*EF1-EF4*) for 30 s, 72°C for 1 min, and a final step of 72°C for 1 min. PCR products were separated by gel electrophoresis and bands were visualized by ethidium bromide staining (Fig. 3.3).

Table 3.2. Amplification and internal sequencing primers used to amplify the two genes studied, including the source of published primers

Gene	Primer	Sequence	Source
EF1α	EF1	ACAGCGACGGTTTGTCTCATGTC	McDonagh and Stevens (2011)
	EF4	CCTGGTTCAAGGGATGGAA	McDonagh and Stevens (2011)
COX1	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

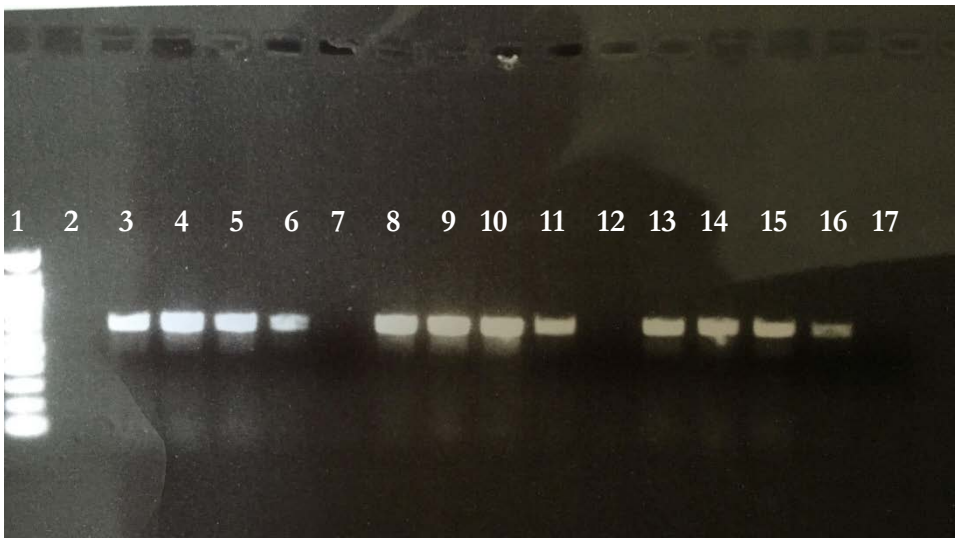

Figure. 3.3. Agarose gel of PCR products for *COX1* from different unidentified larval specimens extracted from toad-myiasis cases. The last lane did not contain any DNA (negative control). 1) 100bp ladder; 2) Control (no template); 3) Lbuf(NLD1); 4) Lbuf(NLD2); 5) Lbuf(NLD3); 6) Lbuf(NLD4); 7) Control (no template); 8) Lbuf(NLD5); 9) Lbuf(NLD6); 10) Lbuf(NLD7); 11) Lbuf(CHE); 12) Control (no template); 13) Lbuf(GBR1); 14) Lbuf(GBR2); 15) Lbuf(GBR3); 16) Lbuf(GBR4); 17) Control (no template).

Table 3.3. Adult *Lucilia* specimens studied, including the identity of the person responsible for morphological identification location of collection, name of sample used for phylogenetic reconstruction, and accession numbers for GenBank DNA sequences for both *COX1* and *EF1a*.

Species	**ID**	**Country/Region of origin**	**Name on Tree**	***COX1***	***EF1α***
Lucilia bufonivora	DM	Baden-Württemberg, Germany	Lbufo(DEU1)	FR719161	FR719238
L. bufonivora	DM	Baden-Württemberg, Germany	Lbufo(DEU2)	FR719161	FR719238
L. bufonivora	GAR	Olst, The Netherlands	Lbufo(Olst)	FR719161	FR719238
L. bufonivora	GAR	Winssen, The Netherlands	Lbufo(WN)	FR719161	FR719238
Lucilia elongata	AT	Canada	Lelongata(CAN)	KM858341*	+LT965032
Lucilia silvarum	GAR	Bristol, UK	Lsilv(GBR1)	KJ394947	FR719260
L. silvarum	GAR	Bristol, UK	Lsilv(GBR2)	KJ394947	FR719260
L. silvarum	GAR	Bristol, UK	Lsilv(GBR3)	KJ394947	FR719260
L. silvarum	RLW	San Francisco, USA	Lsilv(USA)	FR719259*	FR719259*
L. silvarum	RLW	Sacramento, USA	LsilvSacramento	+LT963484	+LT965034
L. silvarum	GAR	Olst, The Netherlands	Lsilv(NLD)	+LT963483	FR719253
Lucilia richardsi	GAR	Bristol, UK	Lrich(1)	FR872384	FR719253
L. richardsi	GAR	Bristol, UK	Lrich(2)	KJ394940	FR719253
Lucilia sericata	GAR	Bristol, UK	Lsericata(UK)	AJ417714	+LT965035
L. sericata	JRS	Los Angeles, USA	Lsericata(US)	AJ417715*	FR719257*
Lucilia cuprina	RLW	Perth, Australia	Lcuprina(AUS)	AJ417707*	FR719245*
L. cuprina	AH/ DMB	Dorie, South Island, New Zealand	Lcuprina(NZL)	AJ417706*	FR719244*
Lucilia caesar	GAR	Bristol, UK	Lcae(Bristol-1)	+LT900367	+LT900482
Lucilia illustris	RLW	Somerset, UK	Lillus	FR872384*	FR719253*
Lucilia ampullacea	GAR	Bristol, UK	Lamp(Bristol)	+LT963485	+LT965033
L. ampullacea	RLW	Somerset, UK	Lamp	FR719236*	EU925394*
Lucilia mexicana	FAV	Chapingo, Mexico	Lmex(Mex1)	+LT900368	+LT900483
L. mexicana	FAV	Chapingo, Mexico	Lmex(Mex2)	+LT900368	+LT900483
*Calliphora vicina^*	GAR	Switzerland (lab. reared)	Cvic(CHE)	KJ635728#	FR719219
C. vicina	GAR	Bristol, UK	Cvic(1)	KJ635728	FR719219
C. vicina	GAR	Bristol, UK	Cvic(2)	KJ635728	FR719219

Adult specimen identification (Table 3.3): GAR=Gerardo Arias-Robledo (Bristol, UK), JRS=Jamie Stevens (Exeter, UK), RLW=Richard Wall (Bristol, UK), FAV=Francisco Arias-

Velazquez (Chapingo, Mexico), DM=Dietrich Mebs (Frankfurt, Germany), AH=Allen Heath (AgResearch, New Zealand), DMB = Dallas Bishop (AgResearch, New Zealand); AT = Angela Telfer (Guelph, Canada). + = new sequence; * = sequence data from EMBL/GenBank; ^ = adult specimen provided by G. Guex (Zurich) thought to be *L. bufonivora*. Identified morphologically at University of Exeter by GAR; # identity confirmed on 540 bp of sequence data of *COX1*.

A negative control (no template DNA) was included in each set of PCR amplifications. Targeted bands of *COX1* were cut out and purified using a QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany). Successful *EF1-EF4* products were purified using 0.5μL of Exonuclease I and 0.5 μL of Antarctic phosphatase per 20 μL of PCR product. A total of **658 bp** of the *COX1* region were amplified in a single fragment with primers HCO2198 and LCO1490. A fragment of **638 bp** of the *EF1a* region was amplified with primers EF1 and EF4. Purified PCR products were sequenced using commercial sequencing facilities, EUROFINS® (*EF1a*) and GENEWIZ® (*COX1*).

3.2.3 Sequence alignment

The quality of the sequences was checked and edited manually for both forward and reverse fragments; sequences were then assembled into a single consensus sequence using BioEdit software (Hall, 1999). Each consensus sequence was checked against previously published sequences in EMBL/GenBank using BLAST. Multiple sequence alignment was carried out using BioEdit implementing the CLUSTALW algorithm.

3.2.4 Phylogenetic analysis

The best-fitting nucleotide substitution model for each dataset was selected using jModelTest (Posada, 2008) (TreNe2f + I was selected for the *EF1-EF4* dataset; TIM3 + I +G was selected for *COX1*). Prior to Bayesian inference analyses the best-fitting model selected for each gene was implemented by changing the default settings (*nst, rates, ngammacat, statefreqpr, revmat, shapepr* and *pinvarpr*) in the software MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001). Phylogenetic analysis was then carried out implementing MCMC starting from two independent analyses simultaneously, each with three heated chains and one cold chain, they were run for 10,000 generations sampling every 10 generations. Analyses were stopped when the critical value for the topological convergence diagnostic fell below the

default threshold (0.01). A fraction (0.25) of the sampled values were discarded (*burninfrac* = 0.25) when the convergence diagnostics were calculated. Substitution model parameters (*sump*) and branch lengths (*sumt*) were summarized; tree topology was then calculated with the remaining data by constructing a majority-rule consensus tree. A combined-gene analysis was also carried out with a partitioned dataset; model parameters for each gene were implemented separately (unlinked), allowing each gene to evolve under different rates. An incongruence length difference test (ILD) was run in PAUP*4.0 (Swofford, 1998) to test phylogenetic congruence and to quantify the differences in topology between the single-gene trees. Analysis was conducted on a partitioned dataset with the combined dataset (*EF1a* and *COX1*).

3.3 Results

3.3.1 Molecular identification of *Lucilia bufonivora*

All 20 larval specimens from the 16 infestations studied gave nuclear and mitochondrial sequence data consistent with BLAST searches for *L. bufonivora*. Additionally, molecular data reaffirmed the identity of adult fly samples identified as *L. bufonivora* on the basis of morphology. All *L. bufonivora* samples were grouped together in a single unstructured clade in all phylogenies (Figs. 3.4 - 3.6).

The adult fly from Switzerland, labelled as '*L. bufonivora*', was identified both morphologically and genetically as *C. vicina* (Table 3.3). This specimen emerged from a 2nd generation of a fly-colony that was originally established from a toad-myiasis case. While it seems that the larvae causing the myiasis wound were *L. bufonivora* (Figs. 3.4 - 3.5), it is likely that they did not survive in laboratory culture without a live-host and were probably outcompeted by *C. vicina*.

3.3.2 Single-gene phylogenies: *EF1α*

All unidentified larval samples found in amphibian myiasis had sequence data of *L. bufonivora*. They were placed in a monophyletic clade along the adult samples from the Netherlands and Germany (Fig. 3.4). Within this group all *L. bufonivora* specimens analysed were classified together in a well-supported clade (Fig. 3.4), with minimal intra-specific variation (only one English specimen from Shrewsbury showed minor variation). However, the analysis did not

show clear distinction of *L. elongata* (a North American species) from *L. silvarum* (Fig. 3.4), although within this group, both USA samples of *L. silvarum* (Sacramento and San Francisco) were placed together with strong support and higher intra-specific variation.

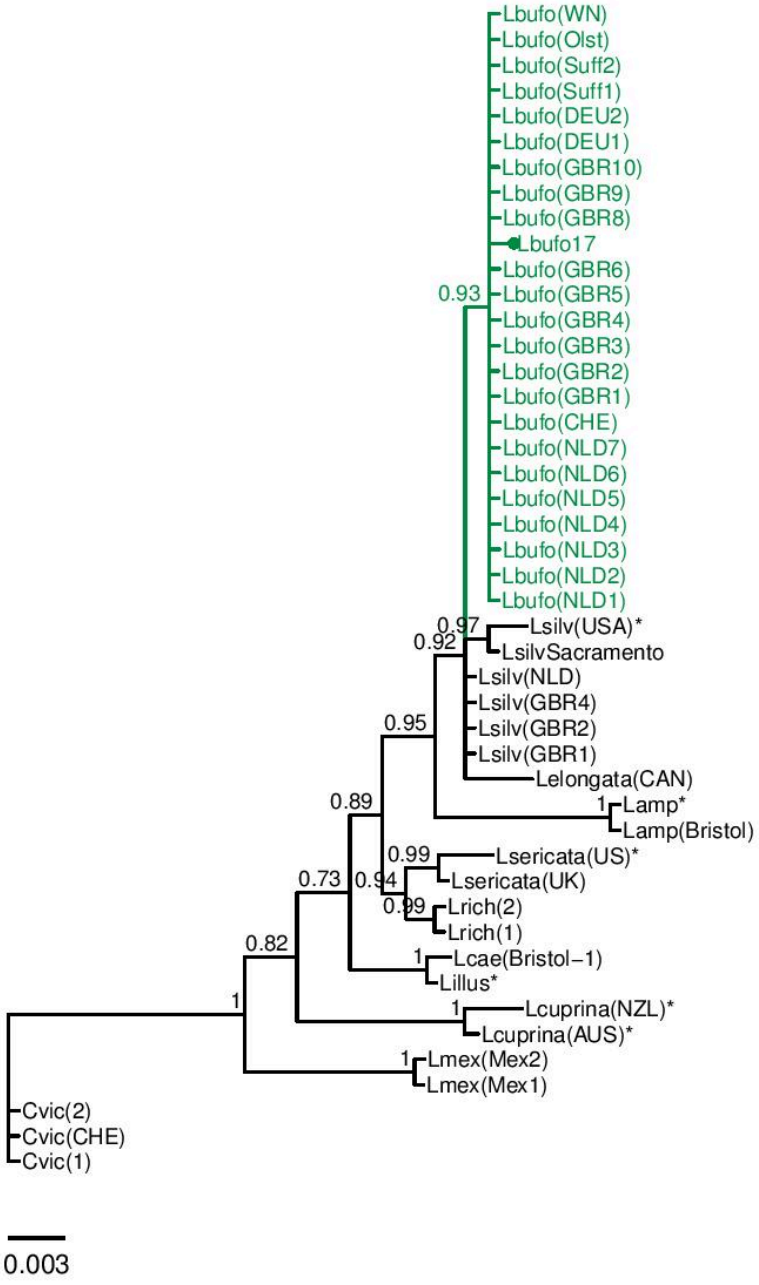

Figure 3.4. Bayesian inference tree constructed from the EF1-EF4 region of the nuclear gene *ef1a*. Green letters correspond to adult and larval samples identified as *Lucilia bufonivora*. Green dot within this clade represents a specimen from Shrewsbury (infestation ID 'Shrew-446') which exhibited minimal intraspecific variation. Posterior probability values are labelled on each node. Scale represents expected changes per site.

Both specimens of *L. ampullacea* were grouped together in a single clade as a sister taxon of the amphibian parasite species group. This analysis also gave strong support to the clear relationships of *L. sericata* and *L. richardsi* (Fig. 3.4), placing together both USA and UK samples of *L. sericata* as a sister clade to the *L. richardsi* clade. *L. caesar* and *L. illustris* were also placed together in a monophyletic group. Both specimens of *L. cuprina* (NZ and AUS) were grouped in a single clade separated from the species mentioned above; a similar pattern of separation was observed with the two sequences of *L. mexicana* (Fig. 3.4). All sequences of *Calliphora vicina* analysed grouped together in the same outgroup clade, with the inclusion of the adult sample misidentified as '*L. bufonivora*' from Switzerland.

3.3.3 Single-gene phylogenies: *COX1*

The Bayesian inference tree based on *COX1* gene sequence data placed all unidentified larval specimens found in toad-myiasis cases in a single clade with the adult samples of *L. bufonivora*. No sequence divergence was detected within this clade. *Lucilia elongata* was grouped as a sister clade to *L. bufonivora* with strong support (Fig. 3.5).

Sequences of *L. richardsi*, a European blowfly species, were placed as a sister clade to the European *L. silvarum* group; however, both North American *L. silvarum* samples were placed apart from this group (*L. richardsi* + European *L. silvarum*), further emphasising the relatively high intra-specific variation in *L. silvarum* (Fig. 3.5).

The Bayesian analysis recovered the sheep myiasis agents *L. sericata* and *L. cuprina* as sister species with strong support (0.99). The *L. caesar* group was also recovered, placing *L. ampullacea* as a sister taxon to the *L. illustris* + *L. caesar* clade. The North American species *L. mexicana* was well separated from the *L. caesar* group. All samples of *C. vicina* used in this study were classified in the same outgroup clade (Fig. 3.5).

3.3.4 Combined-gene phylogeny

The ILD test detected incongruence between the two genes used in this study (P = 0.01); nonetheless, Bayesian inference analysis of a combined partitioned dataset produced a phylogeny with generally strong posterior probabilities (Fig. 3.6). All *L. bufonivora* samples were grouped in a single clade as a sister species to *L. elongata*.

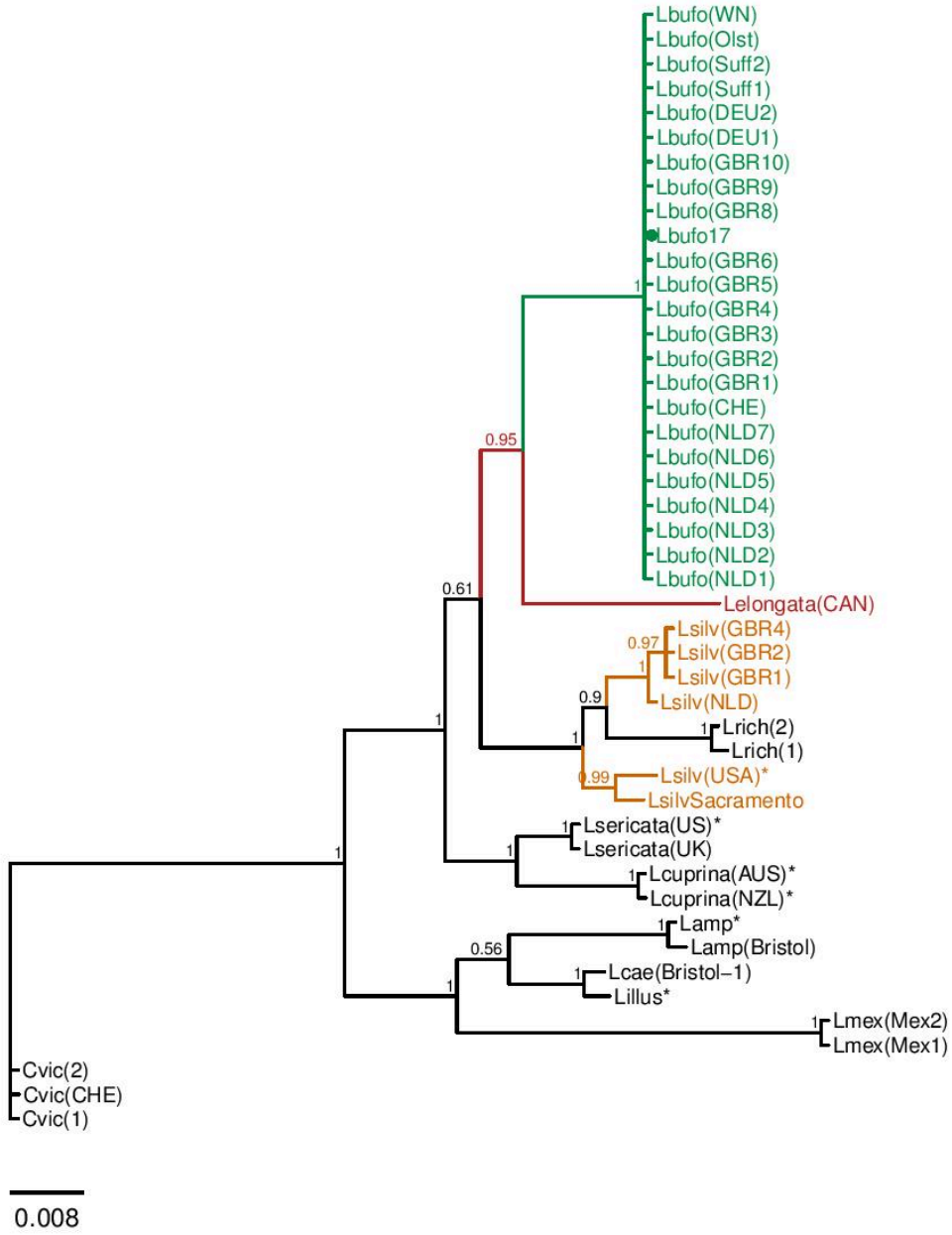

Figure 3.5. Bayesian inference tree constructed from 658bp of *COX1*. Green letters correspond to samples of Lucilia bufonivora, red represents Lucilia elongata and yellow represents Lucilia silvarum. Posterior probability values are labelled on each node. Scale represents expected changes per site.

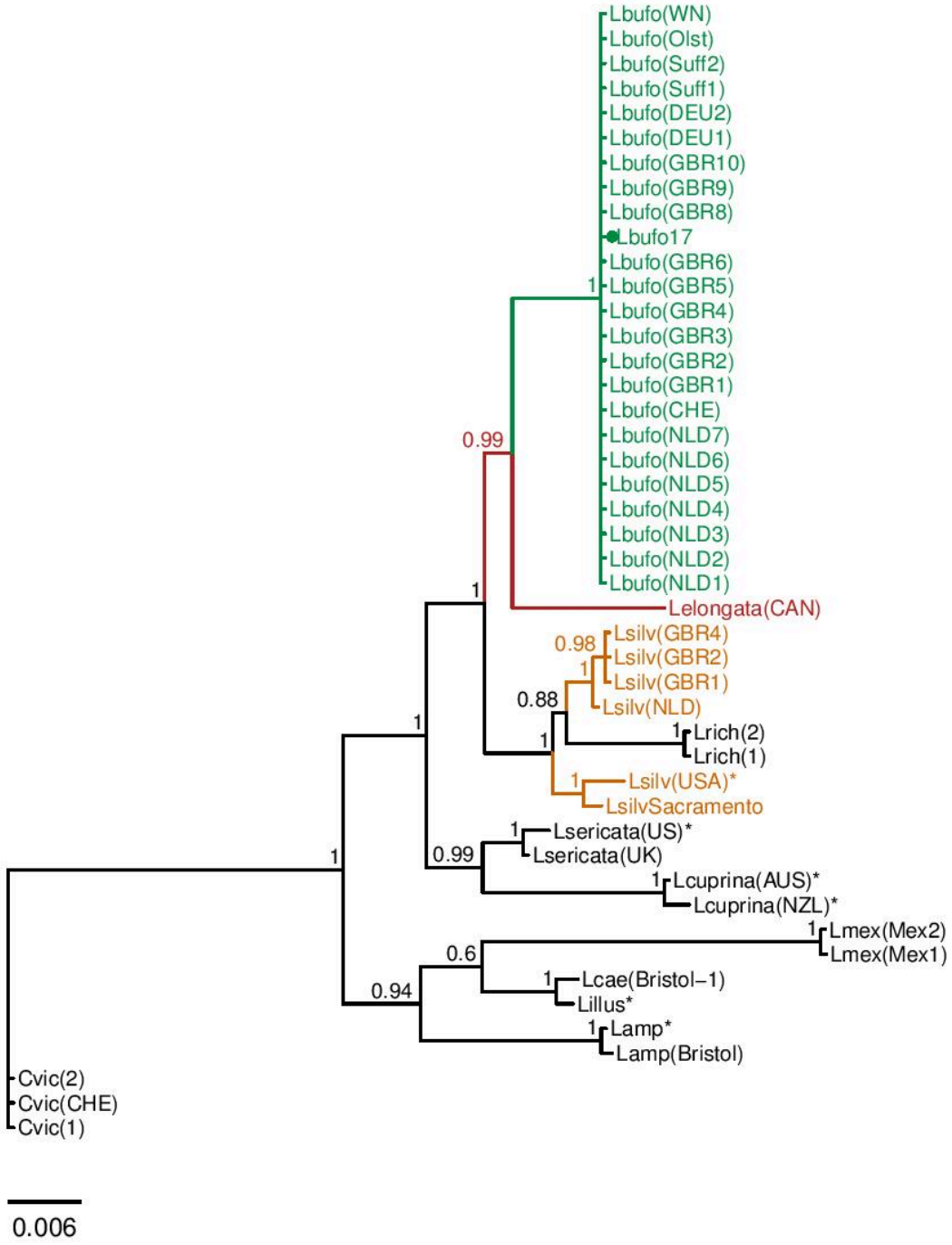

Figure 3.6. Bayesian inference tree constructed from a partitioned dataset of the combined genes *ef1a* and *COX1*. Green letters correspond to samples of *Lucilia bufonivora*, red represents *Lucilia elongata* and yellow represents *Lucilia silvarum*. Green dot within this clade represents a specimen from Shrewsbury (infestation ID 'Shrew-446') which exhibited minimal intraspecific variation. Posterior probability values are labelled on each node. Scale represents expected changes per site.

As observed in the *COX1* tree, a monophyletic European *L. silvarum* group (GBR + NDL) was recovered, with *L. richardsi* grouped as its sister taxon (Fig. 3.6); again, both American specimens of *L. silvarum* were placed outside of this group as sister taxa with high support values. Both sheep blowfly species, *L. sericata* and *L. cuprina*, were recovered as a monophyletic group with strong support. The closely related species *L. illustris* and *L. caesar* were recovered as sister species, however, this combined-gene analysis placed *L. mexicana* more closely related to the *L. caesar* group than the *L. ampullacea* clade. Subfamily relationships of Luciliinae were recovered with strong posterior probability (1), grouping all *C. vicina* samples as an outgroup and differentiating subfamily Calliphorinae from Luciliinae with strong support (Fig. 3.6).

3.4 Discussion

Results presented here suggest that, as hypothesized by Zumpt (1965), the main amphibian myiasis agent in Europe is the toad fly *L. bufonivora*. No specimens of *L. silvarum* were found involved in the amphibian disease; as was previously suggested by some authors (Duncker, 1891; Mortensen, 1892; Linder, 1924; Stadler, 1930). All unidentified larval samples from different countries across Europe were identified as *L. bufonivora* using both nuclear and mitochondrial sequence data.

Amphibians with nasal-myiasis rarely survive infestation (Brumpt, 1934). Larvae keep feeding on the amphibian carcass until they complete their development. Once the host is dead, it can also serve as an oviposition substrate for saprophagous blowflies (e.g. *Calliphora*, etc). A study in Germany found different blowfly species (including *L. sericata* and *C. vicina*) emerging from toad carcasses with signs of nasal-myiasis (Weddeling and Kordges, 2008). Hence, this succession may have generated some of the confusion in relation to species composition in amphibian myiasis. For instance, in the present study, the swiss adult specimen labelled as *'L. bufonivora'* was identified with molecular and morphological data as *C. vicina*. Nonetheless the larval sample extracted from the host before it died had been identified as *L. bufonivora*. This suggests that, as an obligate parasite, *L. bufonivora* did not survive the rearing process in the laboratory culture.

The results from this work resemble those of McDonagh and Stevens (2011), using the mitochondrial *COX1* gene *L. silvarum* and *L bufonivora* were recovered as distinct sister

species. However, in the same study both species were placed in the same clade using *EF1a* and *28S rRNA* as nuclear markers, the latter failing to classify them as distinct species. In this study, the *EF1-EF4* region of the protein-coding nuclear gene *EF1a* showed just a single nucleotide difference between the sequence data of *L. silvarum* and *L. bufonivora*; however, Bayesian inference analysis showed clear groupings, identifying them as distinct sister species. Addition of data from the North American amphibian parasite *L. elongata*, another putatively closely related taxon, allowed an even clearer understanding of the evolutionary relationships between *L. silvarum* and *L. bufonivora*, resulting in the differentiation of them as distinct sister species. The *EF1a* tree supported the suggestion that *L. bufonivora* has diverged relatively recently from its sister taxon *L. silvarum* (Stevens and Wall, 1996). The *COX1*-based phylogeny showed clear relationships and distinction between *L. bufonivora* and *L. silvarum*, a finding reiterated in the combined-gene tree. It is probable that in the combined-gene tree a stronger signal in the mtDNA data (*COX1*) is driving the clear distinction and is dominating the weaker phylogenetic signal of the nuclear data (*EF1-EF4*). The low signal present in the *EF1a* sequence data accords with the lower rate of evolution reported previously in this nuclear gene (McDonagh and Stevens, 2011) compared with that reported in the majority of insect mitochondrial genes (McDonagh et al., 2016). Indeed, *COX1* has been widely used in blowfly systematics (McDonagh and Stevens, 2011; Williams and Villet, 2013; Williams et al., 2016; Yusseff-Vanegas and Agnarsson, 2017) and due to generally higher rates of sequence change in mtDNA it is expected to reach reciprocal monophyly before nuclear genes (Funk and Omland, 2003; Dowton, 2004; Lin and Danforth, 2004). As such, mitochondrial sequence data (e.g. *COX1*) are useful for inferring the relationships of recently diverged species (Stevens and Wall, 1997b; Shao et al., 2001) and the results presented here appear to reaffirm this, suggesting that *L. bufonivora* is clearly a separate sister species to *L. silvarum*. Moreover, species distinctiveness is attributed to both molecular and morphological characters that allow unambiguous identification (Dantas-Torres, 2018). Certainly, employing sequence data of *COX1* combined with morphological data of the adult stage, as indicted by Rognes (1991), identification of *L. bufonivora* can be well performed. This suggests that *L. bufonivora* is a disticnt sister species to *L. silvarum* and was strongly supported by the multi-gene phylogeny. The phylogenetic resolution given from nuclear sequence data, however, is not well clear.

Although the genus '*Bufolucilia*' is still used by some authors (Kraus, 2007; Verves and Khrokalo, 2010; Draber-Mońko, 2013), molecular data presented here suggest that, as

proposed by Rognes (1991), it should be dismissed as a synonym of '*Lucilia*'. For instance, within the *COX1* phylogeny (Fig. 3.5), *L. silvarum* exhibits a closer relationship with *L. richardsi* than with the toad fly, *L. bufonivora*. Given that '*Bufolucilia'* do not include *L. richardsi* (Townsend, 1919; Hall, 1948), it would leave the remainder of the genus *Lucilia* paraphyletic. Moreover, all inferred phylogenies recovered *Lucilia* as a genus with strong posterior values.

Molecular analysis of different populations of *L. bufonivora* from across Europe, detected no intra-specific differences in mitochondrial sequence data, while the nuclear gene *EF1a* exhibited only minimal intra-specific sequence variation (Fig. 3.4). However, in *L. silvarum* marked intra-specific variation in both nuclear and mitochondrial sequence data was observed between European and North American populations of this fly; recent phylogenetic analysis of populations of this species from the USA and Germany also showed a high degree of intra-specific difference (Williams et al., 2016). In the current study, intra-specific variation was also observed between European samples, with UK *L. silvarum* differing from a Dutch specimen of the same species. In contrast, a lack of significant variation in both nuclear and mitochondrial genes in the different European populations of *L. bufonivora* analysed suggests that it may be a recently diverged species that has accumulated less molecular variation. Further studies would be of value, particularly to explore the differences between European and North American populations of *L. bufonivora* (Tantawi and Whitworth, 2014).

Although this study found no specimens of *L. silvarum* involved in amphibian myiasis, in North America there have been several reports of amphibian myiasis cases apparently involving *L. silvarum* (Bolek and Coggins, 2002; Bolek and Janovy, 2004; Eaton et al., 2008). Whether or not this is correct or simply misidentification cannot be determined from the current study. Tantawi and Whitworth (2014) made the first report of *L. bufonivora* in Canada and demonstrated it had been commonly confused with *L. silvarum*. Hence, reports of the latter species causing amphibian myiasis in North America remain arguable and more research is required to further explore the species composition in amphibian myiasis in the Nearctic region.

In England *L. bufonivora* is considered a rare species, nonetheless a relatively recent study have confirmed its involvement in toad myiasis cases (McDonagh and Stevens, 2011). It is rarely caught with traps using standard blowfly baits e.g. carrion (MacLeod and Donnelly,

1956). This may illustrate the highly specific nature of the cues emanating from a living amphibian host that are required to attract *L. bufonivora*, or simply may reflect its restricted distribution and low abundance in the field (or the difficulty associated with its correct morphological identification). Nonetheless, the work presented in this chapter reaffirmed the presence of this obligate parasite in Britain, the Netherlands and Switzerland using larval samples extracted from toad myiasis cases (Fig. 3.3).

European samples of *L. silvarum* appeared to be more closely related to *L. richardsi* than to *L. bufonivora* in the mitochondrial phylogeny (Fig. 3.3). However, the phylogeny constructed with *EF1a* recovered *L. richardsi* as a sister species of *L. sericata,* excluding it from the amphibian parasite group of flies. Similar results have been observed in the past (McDonagh and Stevens, 2011). Although *L. sericata* and *L. silvarum* have been reported as facultative parasites of sheep and amphibians, respectively (Hall, 1948; Zumpt, 1965), another species that exhibited close relationships with them, *L. richardsi*, has never being involved in either sheep or toad myiasis. The high similarity of *L. richardsi* with *L. sericata* based on nuclear DNA and with *L. silvarum* based on mitochondrial DNA, might be due to introgressive hybridization or incomplete lineage sorting. However, there is no robust data to confirm this.

In conclusion, the work presented here suggests that amphibian myiasis in Europe is caused by the toad fly, *L. bufonivora*. Although *L. silvarum* was also thought to also be involved, DNA-based identification methods of unidentified larval specimens showed otherwise. Inferred phylogenies from a nuclear (*EF1a*), a mitochondrial (*COX1*) and a concatenated dataset suggest *L. bufonivora* as a distinct sister species to *L. silvarum*. Recognising the genus '*Bufolucilia*' leaves the remainder of the genus *Lucilia* paraphyletic. A revision of this species-group still needs to be carried out in order to resolve the taxonomic confusion in North America with the inclusion of a broader range of Nearctic samples of *L. bufonivora*, *L. elongata* and *L. silvarum*. Moreover, detailed studies are required to explore the evolution of the obligate parasitic trait within the genus *Lucilia*.

4. Ecological and geographical speciation in blowflies: evolution of an obligate parasite of amphibians

Contributions

Gerardo Arias-Robledo was the main researcher and undertook DNA extractions, primer design, PCR procedures, sequence assembling, editing, statistical analysis and wrote the drafts of the manuscript. Jamie Stevens assisted as main supervisor and supported with manuscript corrections and interpretation of results. Richard Wall supported with general advice, supervision and corrections on the manuscript. Swaid Abdullah provided advice and guidance on primer design. K. Szpila, D. Shpeley, T. Whitworth, J. Memmott, A. Telfer and T. Stark provided samples for analysis. This work was funded by CONACyT and University of Bristol. It was published in **International Journal for Parasitology: Parasites and wildlife.**

4.1 Introduction

Many different lineages of flies within super-family Oestroidea are recognised as parasites of vertebrates. Within Oestroidea, the family Calliphoridae includes a range of saprophagous species, facultative parasites and, to a lesser extent, obligate parasites (Zumpt, 1965; Stevens, et al., 2006) , many of which are of major economic importance in the livestock industry (e.g *Lucilia sericata*, *Lucilia cuprina*, *Cochliomyia hominivorax*). Calliphorid flies typically exhibit low host-specificity, relatively short periods of larval development and are rarely seen infecting hosts in the wild (Erzincliogl u, 1989; Stevens, 2003). Thus, it has been hypothesized that blowflies may have evolved ectoparasitism in association with humans and animal domestication (Stevens and Wall, 1997a; Stevens et al., 2006). However, the toad fly, *Lucilia bufonivora*, is generally associated with wild hosts (Brumpt, 1934; Weddeling and Kordges, 2008). Moreover, it exhibits high host-specificity for amphibians, which is an atypical behaviour for flies in the genus *Lucilia* (Vestjens, 1958; Koskela et al. 1974; Strijbosch, 1980; Gosá et al., 2009; Martín et al., 2012). The life-history of facultative myiasis agents has been well studied in the past due to their economic importance as livestock parasites and as forensic indicators (Zumpt, 1965; Wall et al., 1992a; Stevens, 2003; Wallman et al., 2005). However, information on the evolutionary history of *L. bufonivora* is limited (Stevens and Wall, 1997; Stevens, 2003). Precisely when *L. bufonivora* evolved this high host-specificity for amphibians is unknown. An understanding of this question will contribute to an understanding of the evolution of myiasis in this genus in general and highlight the place in this evolutionary pathway that amphibian myiasis occupies.

It was thought that *L. bufonivora* was a strictly Palearctic species, however Tantawi and Whitworth (2014) recorded adult specimens for the first time in Canada. The latter study, however, used only morphological data and, to date, there are no existing studies of the phylogenetic relationships between Nearctic and Palearctic populations of *L. bufonivora*. Moreover, although adult flies have been reported in North America, studies have not yet confirmed its involvement in amphibian myiasis in this geographical area. Additionally, it is not known whether this constitutes a recent introduction to North America or simply reflects its relative rarity and/or previous taxonomic confusion.

In the United States and Canada, two species have been reported involved in amphibian myiasis: *Lucilia elongata* and *Lucilia silvarum* (Roberts, 1998; Bolek and Coggins, 2002; Bolek and Janovy, 2004). The former is restricted to the Nearctic and has never been observed

breeding in carrion, thus is also considered an obligate parasite of amphibians. In contrast, *L. silvarum* is also distributed throughout the Holarctic (Rognes, 1991; Tantawi and Whitworth, 2014). This species was thought to be involved in amphibian myiasis in Europe (Duncker, 1891; Mortensen, 1892; Linder, 1924; Stadler, 1930), however results from the previous chapter revealed that in Europe amphibian myiasis appears to be caused by exclusively by *L. bufonivora*. Moreover, the saprophagous behaviour of *L. silvarum* has been well documented in the past (Hanski and Kuusela, 1977; Hanski, 1987; Prinkkila and Hanski, 1995; Fremdt et al., 2012). Nevertheless, blowflies often exhibit intraspecific behavioural differences according to their geographical area. As an example, the sheep blowfly *L. sericata* is typically a highly abundant saprophagous species in many countries but behaves as a primary myiasis agent in Northern Europe (Rognes, 1991; Wall et al., 1992a). Hence, variation in the behaviour of *L. silvarum* and its involved in amphibian myiasis could be possible in North America. Molecular analyses may contribute to resolving these issues.

The use of single nuclear DNA molecular markers in isolation such as EF1α does not seem to provide clear phylogenetic resolution of the *L. bufonivora* species group. This has already been observed in the past also with the nuclear rRNA gene 28S (McDonagh and Stevens, 2011). However, the newly optimised nuclear marker, the period gene (*per*), has been used recently to detect hybridization between the closely related sheep blowflies *L. sericata* and *L. cuprina* (Williams and Villet, 2013). Similarly, previous studies have shown that the non-coding Internal Transcribed Spacer 2 (*ITS2*) is a suitable molecular marker for phylogenetic analyses at both genus and species level (Marinho et al., 2011). Thus, the combined use of these molecular nDNA markers might provide a clearer resolution on the relationships of *L. bufonivora*, *L. elongata* and *L. silvarum*.

The aims of this work were, firstly, to infer the times at which the life-history trait of obligate amphibian parasitism arose within a genus that is mainly composed by species with sarco-saprophagous life-cycles. To do this, this work analysed samples from the broad geographical range of *L. bufonivora*, *L. elongata* and *L. silvarum*. Molecular clock-dating was made using a concatenated dataset of a nuclear (per), a mitochondrial (*COX1*) and a non-coding gene (*ITS2*). Secondly, the work aimed to resolve the evolutionary relationships between Palearctic and Nearctic samples of *L. bufonivora* by inferring multiple phylogenies from the genes mentioned above. Finally, the work aimed to provide a better phylogenetic resolution on the *L. bufonivora* species group using relatively recent optimized nuclear markers (e.g. *per* gene).

In addressing these aims, this work offers valuable data on the primers and PCR protocols needed for the successful amplification of a partial sequence of the protein coding *per* gene of *L. bufonivora*. Additionally, it also provides sequence data for blowfly species that have remained understudied due to their low abundance, for example *Lucilia pilosiventris* and *Lucilia regalis*, that could be of importance to forensic entomology. In general, this work highlights the roles of geographical and ecological isolation on the speciation and evolution of blowfly species associated with amphibian myiasis.

4.2 Methods

4.2.1 Biological Material

A total of 43 specimens were analysed in this study. Whole DNA templates from the previous chapter were used to obtain novel sequence data for *ITS2* and *per* genes. New specimens are indicated in Table 4.1.

Twelve specimens of *L. bufonivora* from different locations in Europe were analysed in this study (Table 4.1, Fig. 4.1). Additionally, three *COX1* sequences of the toad fly were obtained from BOLD/Genbank and included in the analysis (three from Canada and one from Spain; Table 4.2)

Eight adult specimens of *L. silvarum* were included, five from different locations in Europe and three from different locations in the USA. Two *COX1* sequences (from Canada and Spain, respectively) were also included in the analysis (Table 4.2)

Lucilia elongata is rarely encountered in the field compared with other blowfly species in North America. This study obtained one specimen from Vancouver, Canada and another one from Alberta (Table 4.1, Fig. 4.1). Two additional *COX1* sequences from the United States and Canada were obtained from BOLD and included in the analysis (Table 4.2).

Figure 4.1 The location of the *COX1* sequences analysed in this study. Red dots represent samples of *Lucilia elongata*, orange dots *Lucilia silvarum* and green dots *Lucilia bufonivora*.

Table 4.1 Specimen list. The table provides the location, name on tree, collector/provider, tissue used for DNA extraction, host (if any), their GenBank/BOLD accession codes for their respective *per*, *ITS2* and *COX1* sequence data, length (bp) of *ITS2* sequences and primers used for the amplification of the *per* gene.

Species	Location	Name on tree	Provided by	Tissue	Host	*per*	*ITS2*	*COX1*	*ITS2* (bp)	*per* primers
Lucilia bufonivora	Winssen, NL	bufonivora_NLWi	G. Arias	Thorax	-	MK062159	MK579385	FR719161	306	3&4
L. bufonivora	Olst, NL	bufonivora_NLOl	G. Arias	Thorax	-	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Roterdam, NL	bufonivora_NLRo	J. Mostert	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Schaopedobe, NL	bufonivora_NLSch	T. Stark	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Wilp, NL	bufonivora_frog	T. Stark	Larva	*Pelophylax kl. esculentus*	MK062158	MK579385	MK598626	306	3&4
L. bufonivora	Norfolk, UK	bufonivora_UKNor	S. Henderson	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Nottingham, UK	bufonivora_UKNot	L. Griffiths	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Shrewsbury, UK	bufonivora_UKShrew	A. Breed	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Ossingen, CHE	bufonivora_CHE	G. Guex	Larva	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Badenw. DEU	bufonivora_DEU	D. Mebs	Thorax↠	*Bufo bufo*	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Borek, POL	bufonivora_POL1	K. Szpila	Thorax	-	MK062160	MK579385	FR719161	306	3&4
L. bufonivora	Czarny Bryńsk, POL	bufonivora_POL2	K. Szpila	Thorax	-	MK062160	MK579385	FR719161	306	3&4
Lucilia silvarum	Bristol, UK	silvarum_UK4	Gerardo Arias	Thorax	-	MK062162	MK579386	KJ394947	312	3&4
L. silvarum	Bristol, UK	silvarum_UK1	G. Arias	Thorax	-	MK062163	MK579386	KJ394947	312	3&4
L. silvarum	Winssen, NL	silvarum_NLWi	G. Arias	Thorax	-	MK062165	MK579386	MK598627	312	3&4
L. silvarum	Olst, NL	silvarum_NLOl	G. Arias	Thorax	-	MK062164	MK579386	LT963483	312	3&4
L. silvarum	Zatwarnica, POL	silvarum_POL	K. Szpila	thorax	-	MN123800	MK579386	KJ394947	312	3&4
L. silvarum	Sacramento, US	silvarum_USACa	J. Stevens	Thorax	-	MK062168	MK579387	LT963484	313	3&4
L. silvarum	Washington, US	silvarum_USAWa	T. Whitworth	Leg	-	MK062166	MK579387	MK598628	313	3&6, 4&5
L. silvarum	Oregon, US	silvarum_USAOr	T. Whitworth	Leg	-	MK062167	MK579387	LT963484	313	3&6, 4&5
Lucilia elongata	Alberta, CAN	elongata_alberta	A. Telfer	Thorax	-	MK062161	MK579388	KM858341	306	3&6, 4&5
L. elongata	Vancouver, CAN	elongata_vancouver	T. Whitworth	Leg	-	MK062161	MK579388	MK598629	306	3&6, 4&5
Lucilia richardsi	Bristol, UK	richardsi_UK1	G. Arias	Thorax	-	MK062169	MK579392	FR872384	333	3&4

L. richardsi	Bristol, UK	richardsi_UK3	G. Arias	Thorax	-	MK062169	MK579392	KJ394940	333	3&4
L. richardsi	Toruń, POL	richardsi_POL	K. Szpila	Thorax	-	MK062169	MK579392	KJ394940	333	3&4
L. pilosiventris	Frankfurt, DEU	pilosiventris_DEU	K. Szpila	Thorax	-	MK598634	MK579397	MK598631	**331**	3&4
L. regalis	Zbocza Płutowskie, POL	regalis_POL	K. Szpila	leg	-	MK598633	MK579396	MK598630	326	3&4
Lucilia caesar	Bristol, UK	caesar_UK	R. Wall	Leg	-	MK062178	MK579393	KM657111	312	1&2
L. caesar	Denizli, TRK	caesar_TRK	K. Szpila	Thorax	-	MK062178	MK579393	KM657111	312	1&2
Lucilia illustris	Olst, NL	illustris_NL	G. Arias	Thorax	-	MK062170	MK579390	KJ394900	314	3&4
Lucilia ampullacea	Bristol, UK	ampullacea_UK	G. Arias	Leg	-	MK062172	MK579391	LT963485	300	1&2
L. ampullacea	Nijmegen, NL	ampullacea_NL	G. Arias	Thorax	-	MK062171	MK579391	LT963485	300	1&2
Lucilia sericata	Chapingo, MX	sericata_MX	F. Arias	Thorax	-	MK062173	EF560187	HQ978732	321	1&2
L. sericata	Winssen, NL	sericata_NL	G. arias	Thorax	-	MK062176	EF560187	AJ417714	321	1&2
L. sericata	Dorset, UK	sericata_UK	J. Memmott	Leg	-	MK062176	EF560187	AJ417714	321	1&2
L. sericata	California, US	sericata_USA	J. Stevens	Leg	-	MK062174	EF560187	HQ978732	321	1&2
L. sericata	Kerman, IRN	sericata_IRN	K. Szpila	Thorax	-	MK598635	EF560187	AJ417714	321	1&2
Lucilia mexicana	Chapingo, MX	mexicana	F. Arias	Thorax	-	MK062177	MK579394	LT900483	331	1&2
Lucilia cuprina	-	cuprina_AUS	-	-	-	JN792783.1	EF560185	AJ417707	335	-
Calliphora vicina	Bristol, UK	Calliphora_UK	G. Arias	Thorax	-	KF839531*	MK579395	FR719219	327	-

New specimens are indicated with bold letters

If no host listed, the samples was collected in its adult stage.

**per* amplification primers: 1&2 = *per*5 and *per*reverse(Williams and Villet, 2013); 3&4 = *pbf*14 and *per*650-R (present study); 3&6= *pbf*14 and *per*433-R (present study); 4&5= *pbf*249 and *per*650-R (present study). New sequence data are shown in red. NOTE Only new sequence data were submitted to GenBank as haplotypes, thus specimens with the same haplotype were allocated with the same accession codes.:

Table 4.2 Additional *COX1* sequences used in this study with their respective location and BOLD/Genbank accession codes.

Species	**Location**	**Accession Code**	**BOLD/GenBank**
Lucilia bufonivora	Spain	GBDP15380-14	BOLD
L. bufonivora	Saskatchewan, CAN	BBDCQ387-10	BOLD
L. bufonivora	Saskatchewan, CAN	CNGSD7561-15	BOLD
L. bufonivora	Saskatchewan, CAN	MF758767.1	Genbank
Lucilia silvarum	Spain	KJ394941.1	Genbank
L. silvarum	Manitoba, CAN	SMTPR3630-16	BOLD
Lucilia elongata	Vancouver, CAN	BBDCP287-10	BOLD
L. elongata	Washington, USA	GMNCF036-12	BOLD
Lucilia richardsi	Germany	GMGMA838-14	BOLD
Lucilia thatuna	California, USA	BBDIT928-11	BOLD
L. thatuna	San Francisco, USA	DQ453489	Genbank

Phylogenetic relationships between the sheep blowflies (*L. sericata* and *L. cuprina*) have been well studied in the past due to their economic importance (Stevens and Wall, 1997b; Wallman et al., 2005; Williams and Villet, 2013). For comparative reasons, this study analysed five *L. sericata* specimens from a broad geographical range (Mexico, United States, the Netherlands, Iran and UK; Table 4.1). All *L. cuprina* sequence data were obtained from Genbank (Table 4.1).

Specimens of *L. richardsi*, *L. regalis*, *L. pilosiventris*, *L. caesar*, *L. illustris*, *L. mexicana* and *L. ampullacea* were also included in the analysis (Table 1). A *C. vicina* specimen from Bristol, UK, was used in the analyses as an outgroup, sequence data for *per* was downloaded from GenBank (Table 4.1). Additionally, two *COX1* sequences of *Lucilia thatuna* (Shannon), another species believed to be implicated in amphibian myiasis in North America (Tantawi and Whitworth, 2014) were included in the analysis (Table 4.2).

4.2.2 Samples of *Lucilia silvarum* associated with amphibian myiasis in Canada

Six single-leg samples labelled as '*Lucilia silvarum*' were provided by D. Shpeley (Assistant Curator, of the insect collection, University of Alberta). All the mentioned samples were collected in Canada, with the earliest sample collected dated 1923 (Table 4.3). Two specimens were reared from diseased amphibians that were collected from different locations in Alberta (Table 4.3).

Table 4.3 *Lucilia silvarum* specimens collected in Alberta, Canada, provided by D. Shpeley including their locality, identifier, year of collection and amphibian host (if the fly was collected by rearing a myiasis case).

Species name	Location	ID by	Year collected	Host (if any)
Lucilia silvarum	Alberta, Toefield	Shannon	1923	-
L. silvarum	Alberta, Lethbridge	Morrison, F.O.	1933	-
L. silvarum	Alberta, Cooking Lake	Hall, D.G.	1937	-
L. silvarum	Alberta, Edmonton	-	1948	-
L. silvarum	Alberta, Pine Lake	Roberts, W.	1991	*Pseudacris triseriata*
L. silvarum	Alberta, Calling lake	Shpeley, D.	1998	*Rana sylvatica*

4.2.3 DNA extractions, primer design and PCR procedures

To avoid contamination, thoracic muscle fibres were extracted from whole adult specimens and used for extractions. In the case of larval specimens, anterior and posterior parts of the larvae where used (or the whole specimen if it was a 1st larval stage) as indicated in Chapter 3. DNA extractions were carried out using a QIAGEN DNeasy®Blood and Tissue Kit (Qiagen GmbH, Germany) according to manufacturer's instructions.

When DNA extraction was undertaken on single legs, muscle fibres were extracted from the trochanter, femur, tibia and, if available, the coxa (Fig. 4.2). This was done by dissecting the legs in ethanol with the aid of a sterile scalpel blade and entomological pins. Once the tissue was extracted it was put in a mix of 80 μL of ATL buffer and 20 μL of Proteinase-K. Cell lysis was carried overnight at 55 °C. In order to increase the yield and concentration of extracted DNA, the elution was carried by adding 40 μL of elution buffer (EB) to the spin-column and it was held for

30 minutes before the spin-down. DNA templates were stored at –20°C for further use. Prior to PCR, the concentration of DNA of each template (ng/ μL) was checked using a Nanodrop.

Amplification of the protein coding gene *per* for various *Lucilia* species was carried out using the primers published by Williams and Villet (2013). Nonetheless, these primers, did not prove suitable for the amplification of this gene in *L. bufonivora* (Fig. 4.3). Therefore, a set of primers (pbf14 and pbf650-R, Table 4.4) was designed for the amplification of ~610bp of the nuclear protein coding gene *per* of the *L. bufonivora* species group (Fig. 4.4). This procedure was carried out using the online software Primer3 v 3.4. (Untergasser et al., 2007) by checking that the difference of melting between primers temperature (TM) was less than 0.5 °C and that each primer had at least 50% or more Guanine-Cytosine base content.

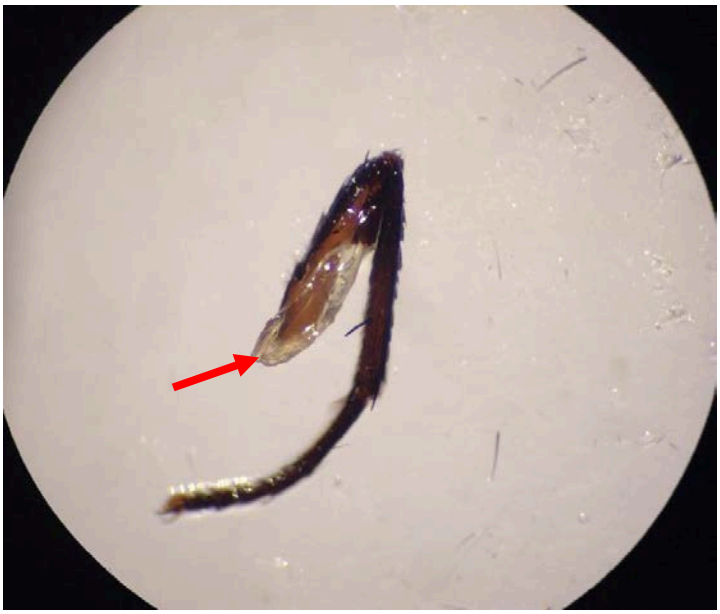

Figure 4.2 Tissue used for DNA extraction. Arrow in red shows the muscle fibres extracted from single-leg samples.

In the case of single leg extractions, an additional set of primers was designed in order to amplify the partial sequence of *per* gene in two fragments of ~410bp each (*pbf*14 + *p*433-R and *p*249 + *pbf*650-R; Table 4.4, Fig. 4.5)

A region of ~650bp of the mitochondrial *COX1* was amplified using the primers LCO1490 and HCO2198 For the amplification of the internal transcribed spacer 2 (*ITS2*) the primers ITS4 and ITS5.8 were use All the primer sequences and their respective PCR protocols are listed in Table 4.4.

PCR products were purified by using 0.5 μL of exonuclease and 0.5 μL of Antarctic phosphatase per 20 μL of PCR product. After purification, both forward and reverse strands were sequenced in the commercial sequencing facilities EUROFINS®.

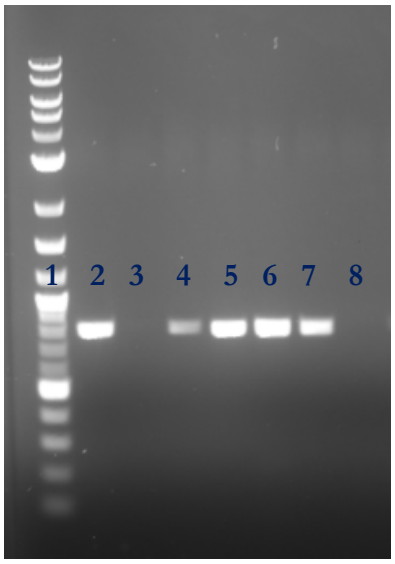

Figure 4.3 Agarose gel of PCR products for *per* gene using primers *per*5' and *per*reverse' (Willams and Villet, 2013). 1) 100bp ladder; 2) *Lucilia sericata* (sericata_UK); 3) *Lucilia bufonivora* (bufonivora_NLWi)*;* 4) *Lucilia silvarum* (silvarum_UK4); 5) *Lucilia caesar* (caesar_UK); 6) *Lucilia ampullacea* (ampullacea_UK); 7) *Lucilia mexicana* (mexicana); 8) control (no template).

4.2.4 Sequence editing and alignment

Forward and reverse chromatograms were checked manually for potential reading errors using BioEdit software (Hall, 1999). The latter software was also used for assembling both strands into a single consensus sequence. Sequences obtained were subject to BLAST searches to confirm species identity. Alignment was done using the ClustalW algorithm in BioEdit (Hall, 1999).

Table 4.4 Primers used for the amplification of *per*, *COX1* and *ITS2*. Name, sequence, source and PCR protocols are described.

				Protocol					
Gene	**Name**	**Sequence**	**Source**	**ID**	**D**	**A**	**E**	**C**	**F**
per	*per*5	GCCTTCAGATACGGTCAAAC	Williams and Villet, 2013	94°C 5min	94°C 30s	50°C 1min	72°C 30s	x36	72°C 7min
	*per*reverse	CCGAGTGTGGTTTGGAGATT							
	*pbf*14	GGCGTTGTCAAGCTCTAGC	this study	94°C 5min	94°C 30s	48°C 1min	72°C 30s	x36	72°C 7min
	*pbf*650-R	CCACGAATGTGAACCAACTC							
	*p*249	GCAAACCAGTAACAGCACCT							
	*p*433-R	GTGCCTGTACCGGTGTTG							
COX1	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994	94°C 5min	95°C 30s	45°C 30s	72°C 1min	x35	72°C 7min
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA							
ITS2	ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990	*94°C 2min	94°C 30s	44°C 35s	72°C 30s	x38	72°C 3min
	ITS5.8	GGGACGATGAAGAACGCAGC							

*ID= initial denaturation step, D=denaturation, A=annealing, E=extension, C= cycles of D-A-E, F=final extension

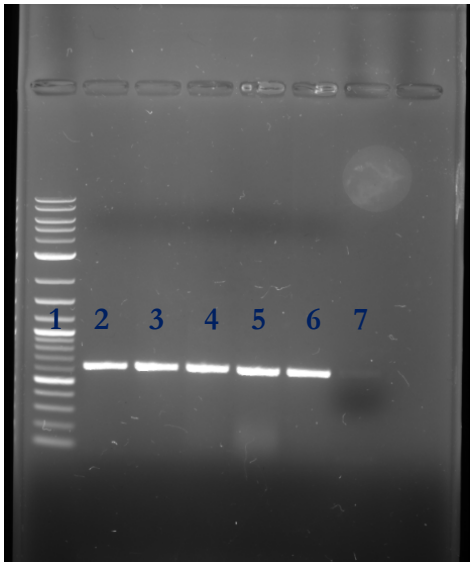

Figure 4.4 Agarose gel of PCR products for *per* gene of *Lucilia bufonivora* amplified with the primers *pbf*14 and *pbf*650-R designed in this study. 1) 100bp ladder; 2) bufonivora_NLWi, bufonivora_NLOl; 3) bufonivora_NLRo; 4) bufonivora_UKNor; 5) bufonivora_DEU; 6) bufonivora_CHE; 7) Control (no template).

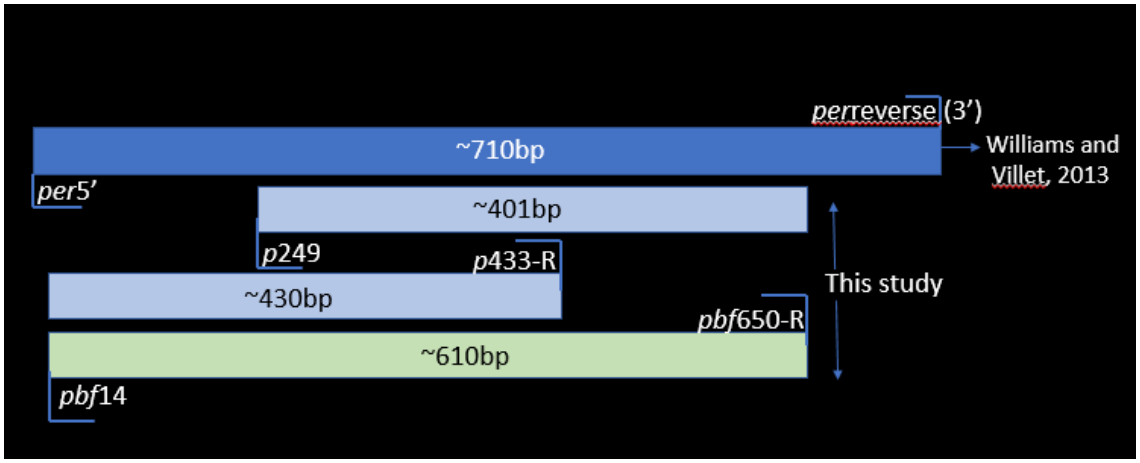

Figure 4.5 Schematic representation of partial region of the protein coding gene *per*, indicating the position, amplifiable sequence length and the name of the primers that were designed in this study as well as the ones published previously by Williams and Villet (2013).

In case of heterozygous sequences (*per*), both forward and reverse chromatograms were checked using BioEdit. Sites that presented two different nucleotide peaks within the same site and with the same height were considered heterozygous sites. Consensus sequences were encoded using their respective IUPAC annotation.

4.2.5 Phylogenetic analyses

Substitution model selection for single-gene datasets was carried out using jModeltest (Posada, 2008) the best-fitting model was chosen using the Bayesian Information Criterion (*ITS2*) and the Akaike Information Criterion (*per*, *COX1*). The models selected were: GTR+F+I+G4 for *COX1*; TIM2+G for *per*; and finally, K3Pu+F+G4 for *ITS2*. In the *ITS2* dataset, gaps were treated as complete deletions. Bayesian inference analysis was done with the software MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001) by implementing the corresponding substitution model to each dataset. A Markov Chain Monte Carlo (MCMC) method was used, starting from two simultaneous independent runs, with three heated chains and one cold chain. Each was run for 10 million generations sampling every thousand generations. When the critical value for the topological convergence diagnostic fell below the default threshold (0.01) analyses were stopped. Burn-in was set to 0.25 to discard a fraction of 0.25 of sampled values. Trees were drawn using R in Rstudio (2015) using the package "ggtree" (Yu et al., 2017). New sequence data were submitted to GenBank (Table 4.1). Pairwise distances of *COX1* were obtained using MEGA7 (Kumar et al, 2006) (Table 4.6). In case of heterozygocity (*per*), sequences were formatted in SeqPHASE (Flot, 2009) and alleles were inferred using PHASE under the default settings.

To provide a clearer resolution to the evolutionary relationships of the *L. bufonivora* group using nuclear DNA, a parsimony splits network of concatenated data set with the inferred *per* alleles and the non-coding *ITS2* was drawn under the default conditions of SplitsTree (Huson and Bryant, 2006).

4.2.6 Divergence time estimation

Tree calibration was done by specifying the node age corresponding to the split between Luciilinae and Calliphorinae subfamilies (19.7 mya) estimated by Wallman et al. (2005) by using an invertebrate mtDNA clock rate of 0.0115 substitutions per site per million years (Brower, 1994). Sequence data for the three genes (mtDNA, nDNA and non-coding) were used for this analysis. Best-fit substitution models were unlinked to allow different evolution rates. Additionally, to allow substitution rates to vary among lineages, the clock model was set to an unlinked log-normal relaxed clock for each gene separately. Clock rate was set to 'estimate' for each dataset under BEAST default settings (Suchard et al., 2018). MCMC consisted of two independent runs, each with a sampling size of 20 million with samples logged every 1000 steps. Convergence between runs was checked using Tracer. Tree files were combined using LogCombiner with a burn-in set to 10%. The software TreeAnnotator from the BEAST package, was used for annotating the maximum credibility tree. The latter was drawn using the package 'strap' (Bell and Lloyd, 2014) using R in Rstudio (2015).

4.3 Results

4.3.1 Specimens of *Lucilia silvarum* implicated in amphibian myiasis in Canada.

Out of the six specimens analysed, DNA was successfully obtained from only two samples; these were collected in 1991 and 1998 and yielded 6.95 ng/uL and 24.7 ng/uL respectively. These adult flies were reared originally from 2 different amphibian myiasis cases in Canada. All three target genes were successfully amplified for these two specimens. Samples from 1937 and 1948 did not yield any amplifiable DNA (Fig. 4.6). No DNA was successfully extracted from the samples from 1923-1933 (0 ng/uL).

Although these samples had been originally labelled as '*L. silvarum*', BLAST searches of *COX1* sequence showed a 100% match to sequence data for *L. bufonivora* from Canada (BBDCQ387-10, Table 4.5). Moreover, with the aid of recent keys (Tantawi and Whitworth, 2014), subsequent morphological identification (carried out by D. Shpeley) supported their identity as *L. bufonivora.*

The latter species was therefore confirmed responsible for the 2 amphibian myiasis cases. in Canada. Thus these samples (originally provided as '*L. silvarum*') were subsequently treated as Canadian *L. bufonivora* in the phylogenetic analyses (Table 4.5).

Table 4.5 Canadian specimens of *Lucilia bufonivora* reared from different amphibian myiasis cases in Alberta. These were originally provided as '*Lucilia silvarum*' (see Table 4.3). Specimens are listed with their location, name on tree, host, Genbank/BOLD accession codes for *per*, *ITS2* and *COX1* as well as sequence length of *ITS2* and the primers used for the amplification of *per* gene.

Species	Location	Name on tree	Host	*per*	*ITS2*	*COX1*	*ITS2* (bp)	*per* primers
Lucilia bufonivora	Pine lake, Alberta	bufonivora_CANPi	*Pseudacris triseriata*	MK598632	MK579389	BBDCQ387-10	304	3&6, 4&5
L. bufonivora	Calling Lake, Alberta	bufonivora_CANCa	*Rana sylvatica*	MK598632	MK579389	BBDCQ387-10	304	3&6, 4&5

per amplification primers: 3&4 = *pbf*14 and *pbf*650-R (present study); 3&6= *pbf*14 and *p*433-R (present study); 4&5= *p*249 and *pbf*650-R (present study). New sequence data are shown in red. Accession codes in blue belong to BOLD database.

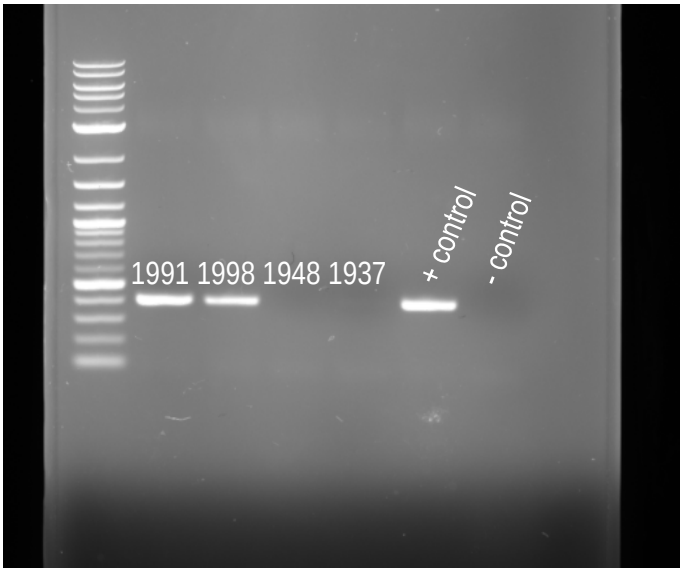

Figure 4.6. Agarose gel of PCR products for a fragment of 410bp of *per* gene. PCR was carried out using DNA from single-leg extractions and using primers *p*249 and *pbf*650-R designed in this work. The first lane is 100bp ladder; second and third lanes belong to Canadian samples of *Lucilia bufonivora*; fourth and fifth lanes to samples provided originally as *Lucilia silvarum*. The date of collection is indicated at the top of their respective band. Sixth lane contains a positive control

with DNA from a European sample of *L. bufonivora* (bufonivora_DEU). Seventh lane is a negative control with no DNA.

4.3.2 Single-gene Phylogenies

Overall, obligate amphibian parasitism was recovered as a monophyletic life history trait in all phylogenies inferred. The saprophagous species *L. silvarum* was never included in this monophyletic group. There was a consistent paraphyly of the toad fly *L. bufonivora* with respect to *L. elongata*, showing a clear distinction between individuals from Europe and Canada. The well-known relationships between the sheep blowflies *L. sericata* and *L. cuprina* were recovered in all phylogenies with strong support. Similarly, the *L. caesar* species group, comprised by mainly saprophagous species very similar in morphology, was supported with strong PPO values in all phylogenies.

ITS2

Amplification of the non-coding region *ITS2* exhibited very variable sequence length among taxa (Table 4.1). Similar to a previous study (Marinho et al., 2011) PCR amplification yielded three different rRNA subunits (5.8S, 2S and 28S), of which subunit 2S splits the region into *ITS2*a (30bp) and *ITS2* (variable length). Since there was very little variation in the *ITS2*a region, phylogenetic analysis included only the 2S, *ITS2* and 28S regions.

Obligate parasitism of amphibians was recovered as a monophyletic trait. This incorporated *L. bufonivora* (Europe), *L. elongata* (North America) and *L. bufonivora* (Canada) (Fig. 4.7). European sequences of *L. bufonivora* exhibited a consistent haplotype with the presence of an indel (8 bp) which was not observed in the Canadian haplotype of the same species (Fig. 4.8). This tree supports the paraphyly of *L. bufonivora* with respect to *L. elongata*. Whilst exhibiting generally lower posterior values, this tree recovered a European *L. silvarum* clade that was distinct from a North American clade of the same species (Fig. 4.7).

This phylogeny recovered with strong support a *L. sericata* species group, that included both economically important sheep blowfly species, *L. sericata* and *L. cuprina*, as well as the morphologically similar species *L. pilosiventris*/*L. regalis* and *L. richardsi*. Despite the geographical distances, this phylogeny recovered a single monophyletic clade of the British sheep blowfly *L. sericata* incorporating samples from United States, Mexico, the Netherlands, Iran and UK (Fig. 4.7).

Similarly, this BI phylogeny recovered the monophyly of the *L. caesar* species group with strong support. *Lucilia caesar* from Turkey and UK did not exhibit any intraspecific variation. Although *L. caesar* and *L. illustris* were grouped as sister species, they showed very short divergence distances between each other, highlighting the close relationship of these species. *Lucilia ampullacea* was recovered as a sister clade to *L. caesar*/*L. illustris*. And, finally, the strictly Nearctic species *L. mexicana* was recovered as a sister species to the *L. caesar* species group (Figure 4.7).

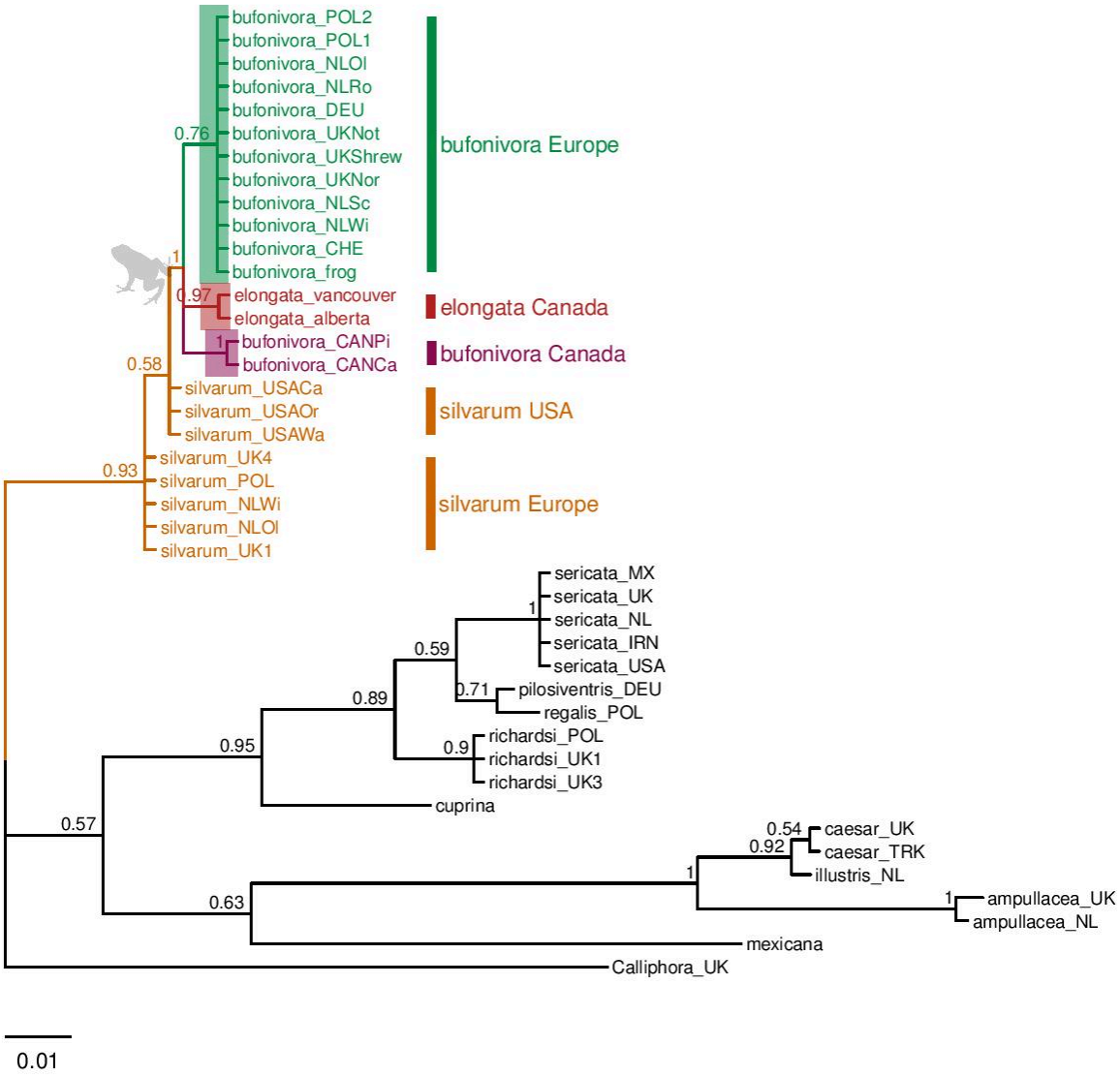

Figure 4.7 BI tree constructed from Internal transcribed Spacer 2 (non-coding). Each specimen is labelled with the species name and location abbreviation as indicated in Table 4.1 and 4.5. Posterior probability values are labelled on each node. Scale represents expected changes per site. Green letters correspond to European samples of *Lucilia bufonivora*; red represents *Lucilia elongata*; purple represents Canadian *L. bufonivora* and finally yellow represents *L. silvarum*.

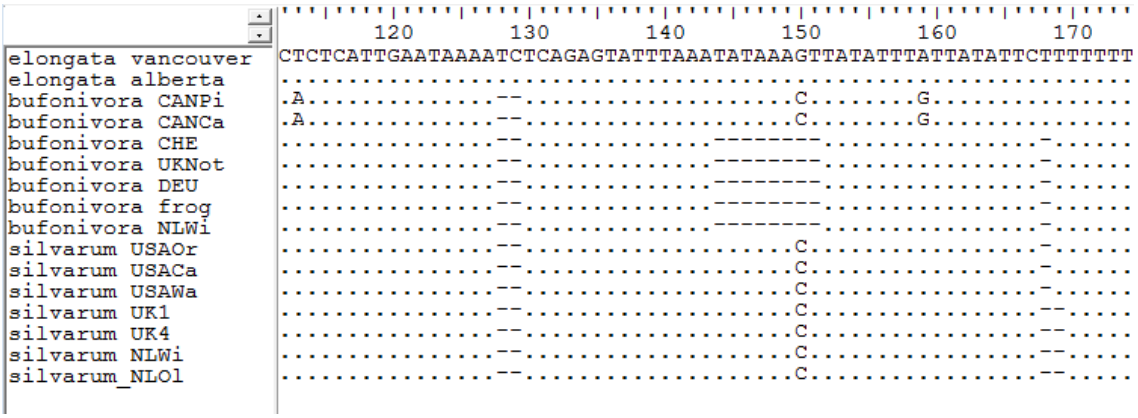

Figure 4.8 Alignment of partial *ITS2* sequences of *L. bufonivora* species-group taxa. Matching nucleotides with *Lucilia elongata* (MK579388) are represented with a dot. Sequence gaps are shown as hyphens. Nucleotide positions are shown above the alignment. Location abbreviations are quoted as indicated in Tables 4.1 and 4.5.

COX1

Bayesian inference analysis recovered all taxa known to be obligate parasite of amphibians in a monophyletic group with strong support (Fig. 4.9). Within this group there is a well-supported Palearctic clade with the inclusion of all European samples of *L. bufonivora*. Nonetheless, all Canadian samples of *L. bufonivora* were clustered together in a single clade independent from their European conspecifics. This clade included three additional sequences from Canada obtained from BOLD/Genbank. Samples of the strictly Nearctic *L. elongata*, although with some intraspecific variation, were recovered as a monophyletic sister clade to the Canadian clade of *L. bufonivora*. Therefore, *L. bufonivora* was recovered paraphyletically with respect to *L. elongata*. Pairwise genetic distances between European and Canadian sequences of *L. bufonivora* were rather high (0.052, TTable 4.6).

The saprophagous species *L. silvarum* was grouped outside of the monophyletic group of species implicated in amphibian myiasis (*L. bufonivora* + *L. elongata*). It was recovered as a paraphyletic species with respect to *L. richardsi*/*L. pilosiventris*/*L. regalis* with strong support. Samples from the Netherlands, UK, Spain and Poland were grouped in a single 'European' clade separate from a North American clade which incorporated all samples from Canada and North America (Fig. 4.9).

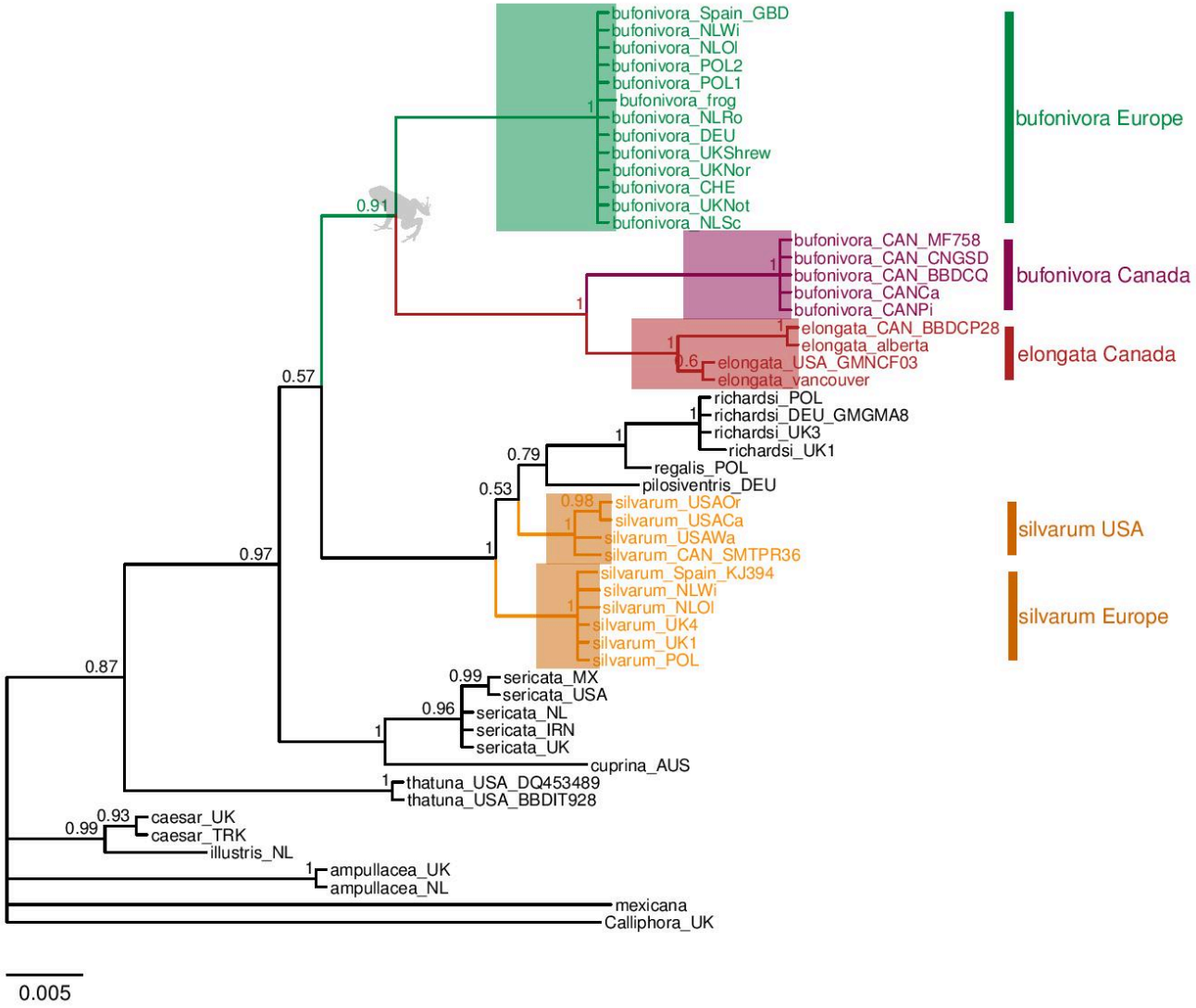

Figure 4.9 Bayesian Inference tree constructed from *COX1* (mtDNA). Each specimen is labelled with the species name and location abbreviation as indicated in Tables 4.1, 4.2 and 4.3. Sequences obtained from BOLD/GenBank are also annotated with their respective accession codes. Posterior probability values are labelled on each node. Scale represents expected changes per site. Green letters correspond to European samples of *Lucilia bufonivora*; red represents *Lucilia elongata*; purple represents Canadian *L. bufonivora* and finally yellow represents *Lucilia silvarum*.

L. richardsi, L. pilosiventris and *L. regalis* showed close relationships to one another (Fig. 4.9). Despite their close relationship to *L. sericata,* this analysis placed them as a sister group to the North American *L. silvarum* clade. This resembles to previous results from a morphological-based parsimony analysis (Stevens and Wall, 1996).

The sheep blowfly, *L. sericata*, was recovered as a monophyletic taxon with strong support incorporating sequences from UK, the Netherlands, Iran, North America and Mexico. Despite the geographical distances, minimal intraspecific variation was detected within this clade. The Australian sheep blowfly *L. cuprina* grouped next to the *L. sericata* clade with strong support (Fig. 4.9). The pairwise distance displayed between them was lower than the one observed between the Canadian and European *L. bufonivora* (0.022, Table 4.6).

Lucilia thatuna, another species that is thought to be implicated in amphibian myiasis in North America (Tantawi and Whitworth, 2014), does not have close evolutionary relationships with the *L. bufonivora* species group (Fig. 4.9).

per

Species implicated in amphibian myiasis were recovered as a monophyletic group with strong support (Fig. 4.10). This group incorporated three distinct clades: *L. bufonivora* (Europe), *L. elongata* (Canada) and another *L. bufonivora* (Canada). Thus, this phylogeny also supported the paraphyly of *L. bufonivora* with respect to *L. elongata*. Unlike previous phylogenies, all samples of *L. silvarum* (both European and North American) were grouped in a single clade with strong support. Outside of this group, *L. pilosiventris*, was recovered as a sister clade showing close relationships with to *L. silvarum*. This is in contrast with previous phylogenies where *L. pilosiventris* showed close relationships with *L. richardsi* and *L. regalis*. These results match with previous morphological analyses (Stevens and Wall, 1996).

Nevertheless, in this phylogeny *L. richardsi* showed close relationships with *L. regalis*, and they were recovered as a sister group to a sheep blowfly group (*L sericata* + *L. cuprina*). All samples of *L. sericata* were grouped in a monophyletic clade, with minimal variation displayed from samples of the American continent (Fig. 4.10). The Australian sheep blowfly, *L. cuprina*, was recovered as a sister species to *L. sericata* with strong support (Fig. 4.10).

L. caesar, *L. illustris* and *L. ampullacea* together formed a monophyletic *L. caesar* group with strong support. The degree of divergence between the sister species *L. illustris* and *L. caesar* was higher than that observed in the *ITS2* phylogeny (Fig. 4.10).

Table 4.6 The pairwise genetic distances computed with *COX1* sequence data of various *Lucilia* specimens. The green circle highlights the distance between European and Canadian *Lucilia bufonivora*. The red circle highlights the distance between *Lucilia sericata* and *Lucilia cuprina*.

	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
4. bufonivora CHE																																		
5. bufonivora UKNor																																		
6. bufonivora UKShrew	0.000																																	
7. bufonivora DEU	0.000	0.000																																
8. bufonivora NLRo	0.000	0.000	0.000																															
9. bufonivora frog	0.002	0.002	0.002	0.002																														
10. bufonivora NLOl	0.000	0.000	0.000	0.000	0.002																													
11. bufonivora NLWi	0.000	0.000	0.000	0.000	0.002	0.000																												
12. bufonivora Spain GBD	0.000	0.000	0.000	0.000	0.002	0.000	0.000																											
13. bufonivora CANPi	0.052	0.052	0.052	0.052	0.051	0.052	0.052	0.052																										
14. bufonivora CANCa	0.052	0.052	0.052	0.052	0.051	0.052	0.052	0.052	0.000																									
15. bufonivora CAN BBDCQ	0.052	0.052	0.052	0.052	0.051	0.052	0.052	0.052	0.000	0.000																								
16. bufonivora CAN CNGSD	0.052	0.052	0.052	0.052	0.051	0.052	0.052	0.052	0.000	0.000	0.000																							
17. bufonivora CAN MF758	0.052	0.052	0.052	0.052	0.051	0.052	0.052	0.052	0.000	0.000	0.000	0.000																						
18. elongata vancouver	0.042	0.042	0.042	0.042	0.040	0.042	0.042	0.042	0.026	0.026	0.026	0.026	0.026																					
19. elongata alberta	0.045	0.045	0.045	0.045	0.043	0.045	0.045	0.045	0.034	0.034	0.034	0.034	0.034	0.011																				
20. elongata CAN BBDCP28	0.045	0.045	0.045	0.045	0.043	0.045	0.045	0.045	0.034	0.034	0.034	0.034	0.034	0.011	0.000																			
21. elongata USA GMNCF03	0.042	0.042	0.042	0.042	0.040	0.042	0.042	0.042	0.026	0.026	0.026	0.026	0.026	0.000	0.011	0.011																		
22. silvarum UK1	0.047	0.047	0.047	0.047	0.045	0.047	0.047	0.047	0.060	0.060	0.060	0.060	0.060	0.053	0.051	0.051	0.053																	
23. silvarum UK4	0.047	0.047	0.047	0.047	0.045	0.047	0.047	0.047	0.060	0.060	0.060	0.060	0.060	0.053	0.051	0.051	0.053	0.000																
24. silvarum NLOl	0.045	0.045	0.045	0.045	0.043	0.045	0.045	0.045	0.058	0.058	0.058	0.058	0.058	0.055	0.053	0.053	0.055	0.002	0.002															
25. silvarum NLWi	0.045	0.045	0.045	0.045	0.043	0.045	0.045	0.045	0.058	0.058	0.058	0.058	0.058	0.051	0.049	0.049	0.051	0.002	0.002	0.003														
26. silvarum CAN SMTPR36	0.040	0.040	0.040	0.040	0.042	0.040	0.040	0.040	0.064	0.064	0.064	0.064	0.064	0.056	0.055	0.055	0.056	0.016	0.016	0.014	0.014													
27. silvarum Spain KJ394	0.045	0.045	0.045	0.045	0.043	0.045	0.045	0.045	0.059	0.059	0.059	0.059	0.059	0.051	0.049	0.049	0.051	0.002	0.002	0.003	0.003	0.014												
28. silvarum USACa	0.040	0.040	0.040	0.040	0.042	0.040	0.040	0.040	0.064	0.064	0.064	0.064	0.064	0.056	0.055	0.055	0.056	0.016	0.016	0.014	0.014	0.003	0.014											
29. silvarum USAOr	0.040	0.040	0.040	0.040	0.042	0.040	0.040	0.040	0.064	0.064	0.064	0.064	0.064	0.056	0.055	0.055	0.056	0.016	0.016	0.014	0.014	0.003	0.014	0.000										
30. silvarum USAWa	0.040	0.040	0.040	0.040	0.041	0.040	0.040	0.040	0.062	0.062	0.062	0.062	0.062	0.056	0.055	0.055	0.056	0.016	0.016	0.014	0.014	0.003	0.014	0.003	0.003									
31. thatuna USA BBDIT928	0.059	0.059	0.059	0.059	0.057	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.057	0.061	0.061	0.057	0.058	0.058	0.056	0.056	0.062	0.057	0.062	0.062	0.058								
32. thatuna USA DQ453489	0.059	0.059	0.059	0.059	0.057	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.057	0.061	0.061	0.057	0.058	0.058	0.056	0.056	0.062	0.057	0.062	0.062	0.058	0.000							
33. richardsi UK1	0.047	0.047	0.047	0.047	0.045	0.047	0.047	0.047	0.070	0.070	0.070	0.070	0.070	0.057	0.059	0.059	0.057	0.022	0.022	0.021	0.021	0.022	0.021	0.022	0.022	0.022	0.063	0.063						
34. richardsi UK3	0.049	0.049	0.049	0.049	0.047	0.049	0.049	0.049	0.068	0.068	0.068	0.068	0.068	0.055	0.057	0.057	0.055	0.021	0.021	0.019	0.019	0.021	0.019	0.021	0.021	0.021	0.061	0.061	0.002					
35. richardsi UKADA1	0.047	0.047	0.047	0.047	0.045	0.047	0.047	0.047	0.070	0.070	0.070	0.070	0.070	0.057	0.059	0.059	0.057	0.022	0.022	0.021	0.021	0.022	0.021	0.022	0.022	0.022	0.063	0.063	0.000	0.002				
36. richardsi DEU GMGMA8	0.049	0.049	0.049	0.049	0.047	0.049	0.049	0.049	0.068	0.068	0.068	0.068	0.068	0.055	0.057	0.057	0.055	0.021	0.021	0.019	0.019	0.021	0.019	0.021	0.021	0.021	0.061	0.061	0.002	0.000	0.002			
37. cuprina AUS	0.049	0.049	0.049	0.049	0.047	0.049	0.049	0.049	0.058	0.058	0.058	0.058	0.058	0.051	0.060	0.060	0.051	0.045	0.045	0.043	0.043	0.045	0.047	0.045	0.045	0.045	0.055	0.055	0.060	0.058	0.060	0.058		
38. sericata UK	0.038	0.038	0.038	0.038	0.036	0.038	0.038	0.038	0.049	0.049	0.049	0.049	0.049	0.039	0.046	0.046	0.039	0.040	0.040	0.038	0.038	0.040	0.038	0.040	0.040	0.040	0.040	0.040	0.043	0.042	0.043	0.042	0.022	
39. sericata NL	0.038	0.038	0.038	0.038	0.036	0.038	0.038	0.038	0.049	0.049	0.049	0.049	0.049	0.039	0.046	0.046	0.039	0.040	0.040	0.038	0.038	0.040	0.038	0.040	0.040	0.040	0.040	0.040	0.043	0.042	0.043	0.042	0.022	0.000
40. sericata USA	0.040	0.040	0.040	0.040	0.038	0.040	0.040	0.040	0.051	0.051	0.051	0.051	0.051	0.040	0.048	0.048	0.040	0.042	0.042	0.040	0.040	0.042	0.040	0.042	0.042	0.042	0.042	0.042	0.045	0.044	0.045	0.044	0.024	0.002
41. sericata MX	0.040	0.040	0.040	0.040	0.038	0.040	0.040	0.040	0.051	0.051	0.051	0.051	0.051	0.040	0.048	0.048	0.040	0.042	0.042	0.040	0.040	0.042	0.040	0.042	0.042	0.042	0.042	0.042	0.045	0.044	0.045	0.044	0.024	0.002
42. illustris NL	0.070	0.070	0.070	0.070	0.068	0.070	0.070	0.070	0.068	0.068	0.068	0.068	0.068	0.070	0.072	0.072	0.070	0.068	0.068	0.066	0.066	0.068	0.066	0.068	0.068	0.068	0.044	0.044	0.077	0.075	0.077	0.075	0.054	0.052
43. caesar UK	0.064	0.064	0.064	0.064	0.062	0.064	0.064	0.064	0.058	0.058	0.058	0.058	0.058	0.064	0.066	0.066	0.064	0.062	0.062	0.060	0.060	0.062	0.060	0.062	0.062	0.062	0.046	0.046	0.074	0.072	0.074	0.072	0.052	0.054
44. ampullacea NL	0.068	0.068	0.068	0.068	0.070	0.068	0.068	0.068	0.079	0.079	0.079	0.079	0.079	0.080	0.076	0.076	0.080	0.076	0.076	0.074	0.074	0.066	0.074	0.070	0.070	0.070	0.068	0.068	0.089	0.087	0.089	0.087	0.078	0.065
45. ampullacea UK	0.068	0.068	0.068	0.068	0.070	0.068	0.068	0.068	0.079	0.079	0.079	0.079	0.079	0.080	0.076	0.076	0.080	0.076	0.076	0.074	0.074	0.066	0.074	0.070	0.070	0.070	0.068	0.068	0.089	0.087	0.089	0.087	0.078	0.065
46. mexicana	0.100	0.100	0.100	0.100	0.098	0.100	0.100	0.100	0.098	0.098	0.098	0.098	0.098	0.095	0.097	0.097	0.095	0.102	0.102	0.100	0.100	0.107	0.100	0.107	0.107	0.102	0.078	0.078	0.106	0.104	0.106	0.104	0.103	0.093

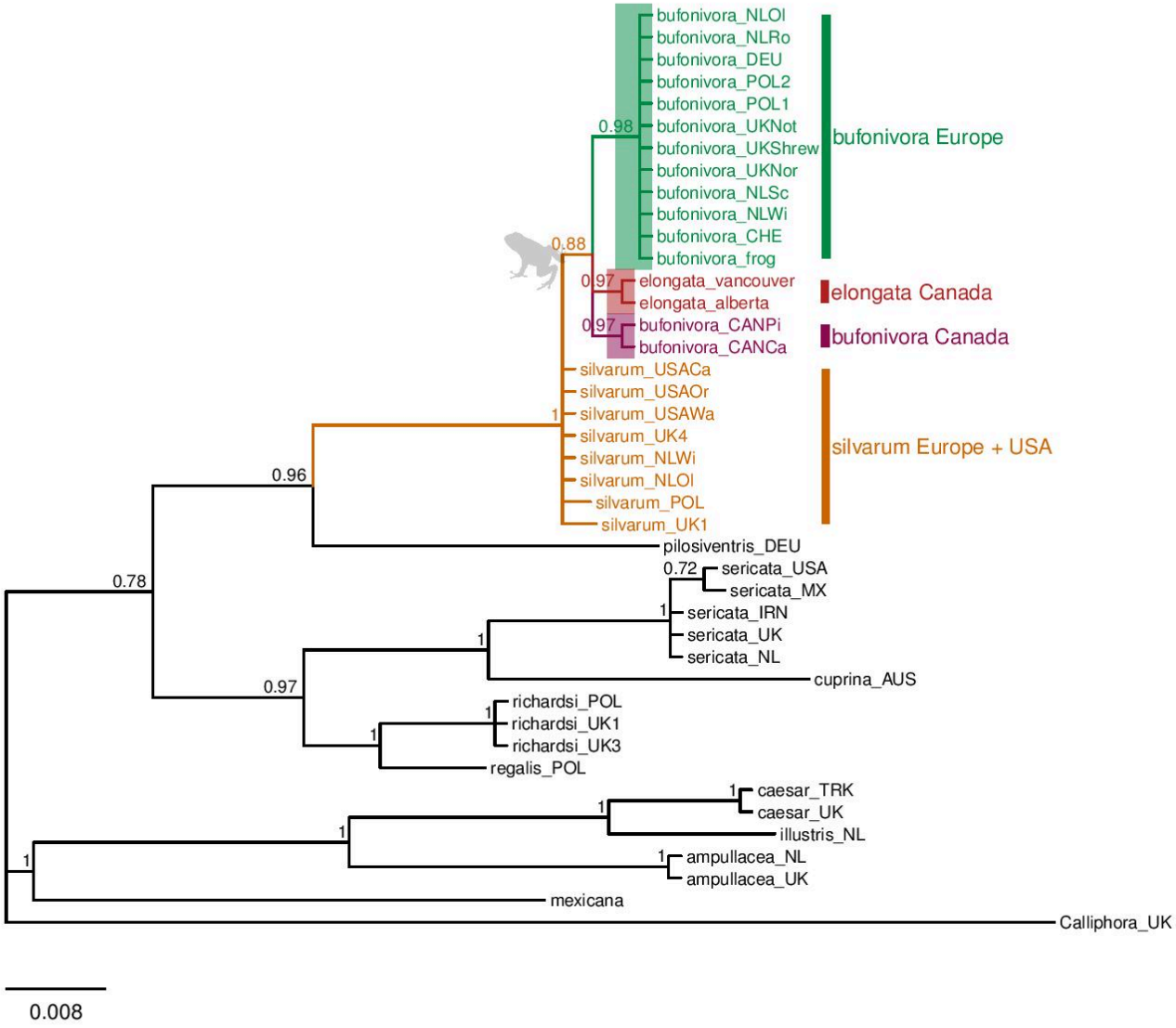

Figure 4.10 BI tree construted from *per* gene (nDNA). Each specimen is labelled with the species name and location abbreviation as indicated in Tables 4.1 and 4.5. Posterior probability values are labelled on each node. Scale represents expected changes per site. Green letters correspond to European samples of *Lucilia bufonivora*; red represents *Lucilia elongata*; purple represents Canadian *L. bufonivora* and finally yellow represents *Lucilia silvarum*.

4.3.3 Parsimony splits: *ITS2* + *per*

A concatenated data set of *per* and *ITS2* gene sequence data resulted in the analysis of ~1050bp (the number of bp is not exact due to the highly variable sequence length of *ITS2*)(Table 4.1). As suggested previously by the *COX1* phylogeny, parsimony splits of the concatenated dataset clustered two well separated groups of amphibian parasites: a Nearctic (*L. elongata* and Canadian *L. bufonivora*) and a Palearctic (European *L. bufonivora*). Both displayed almost the same genetic distance with respect to the *L. silvarum* cluster (Fig. 4.11).

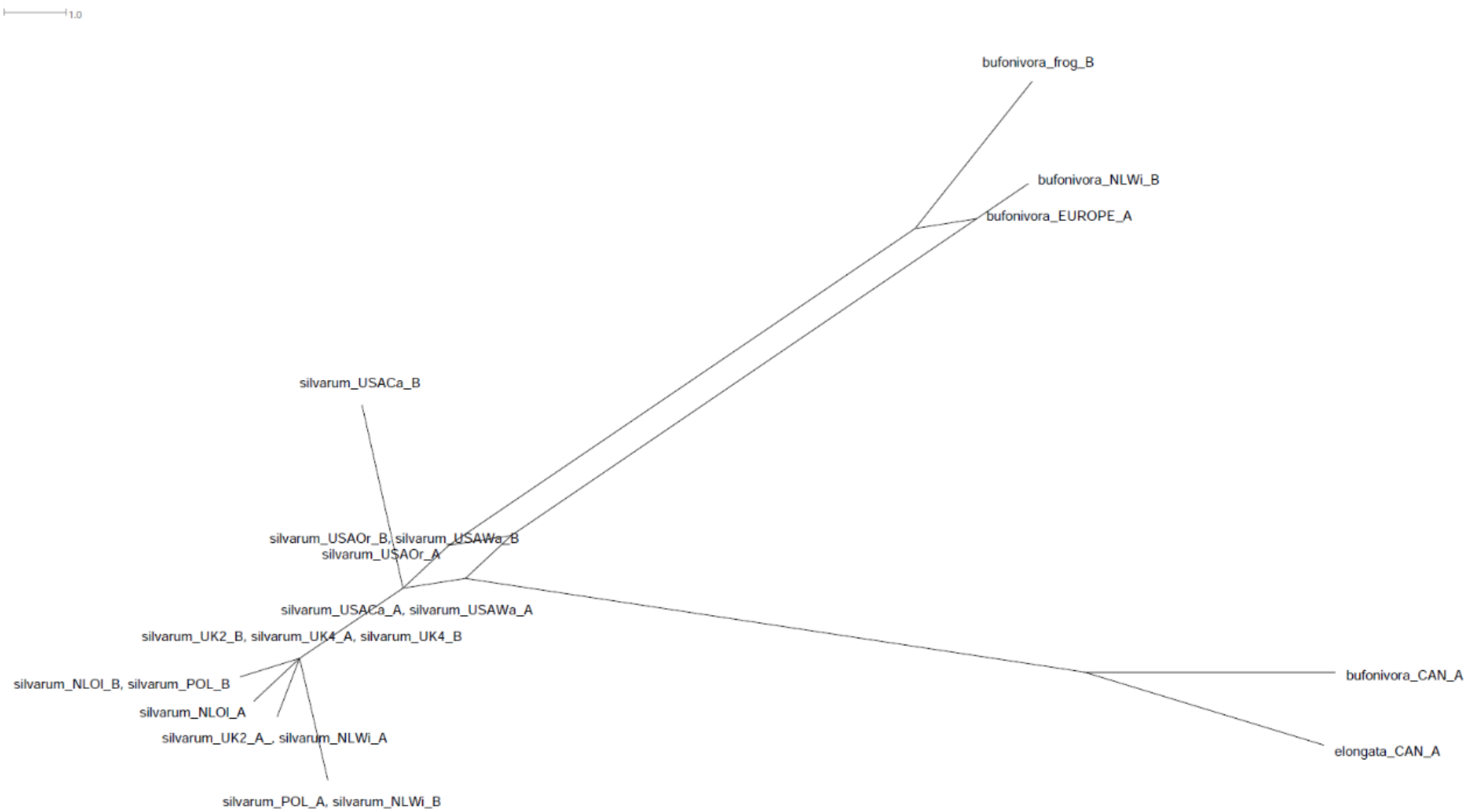

Figure 4.11 Parsimony splits network constructed from a *per* and *ITS2* concatenated dataset. Heterozygous specimens are indicated with A and B. 'bufonivora_EUROPE_A' is composed by a consistent haplotype present in all 12 samples from Europe (Table 4.1), of which just two were heterozygous ('bufonivora_frog' and 'bufonivora_NLWi'). 'bufonivora_CAN' and 'elongata_CAN' are represented by two samples each, none of them were heterozygous. Scale represents expected changes per site.

4.3.4 Divergence time estimation

A concatenated dataset of *COX1*, *ITS2* and *per* (~1700 bp) was analysed. A Bayesian uncorrelated relaxed clock was used to estimate the divergence time for a range of different species of *Lucilia*. The molecular clock calibration was set to the split between the subfamilies Luciliinae and Caliphorinae which was estimated to happen around 19.7mya (Wallman et al., 2005). The present estimates indicate that the main radiation of the genus *Lucilia* occured during the middle Miocene about 15.57mya (95%CI: 10.69-20.26mya, Figure 4.12). Results match with previous estimations on the diversification of genera within Calliphoridae (Junqueira et al., 2016; Wallman et al., 2005). Present results suggest that during this time, there was a major splitting between a lineage of mostly saprophagous habits (*L. caesar* group) and a lineage that would include the sheep blowfly (*L. sericata*) and the toad fly (*L. bufonivora*) species-groups (Figure 4.12).

The split between the *L. bufonivora* and the *L. sericata* species-groups was inferred to have occurred during the Miocene around 9.26mya (95%CI: 5.6-13.10mya, Figure 4.12). This suggests that the *L. bufonivora* group may have diverged from a saprophagous ancestor. Diversification of the *L. bufonivora* group was estimated to have occurred during the Pliocene Epoch 4.98mya (CI: 1.92-8.4mya, Fig. 4.12). Within this group, niche isolation of their most recent ancestor might have played an important role in the adaptative radiation of two well distinct lineages: one with saprophagous behaviour (*L. silvarum*) and another one that evolved high host-specificity for amphibians (*L. bufonivora* + *L. elongata*).

This group of taxa suffered fast diversification that seemed to be driven by geographical barriers. For instance, present results suggest that the diversification of the most recent ancestor to *L. bufonivora* was facilitated by geographical isolation between Nearctic and Palearctic individuals, which was estimated to have occurred 3.52mya (95%CI: 1.08-6.35mya, Fig. 4.12). Certainly, in Europe it diverged into a well-defined Palearctic *L. bufonivora*. However, in North America it diverged into a Nearctic lineage that diversified 1mya later into *L. elongata* and a Nearctic *L. bufonivora* (2.19mya, 95%CI: 0.5-4.02mya, Fig. 4.12). Therefore, the latter species has been present in the North American continent since then but has remained unrecorded possibly due to its low abundance and/or taxonomic confusion.

Similarly, the divergence between a Nearctic and Palearctic *L. silvarum* was inferred to occur 3.05mya (95%CI: 0.8-5.02mya, Fig. 4.12). This also suggests the independent evolution of this saprophagous species between two geographically isolated populations.

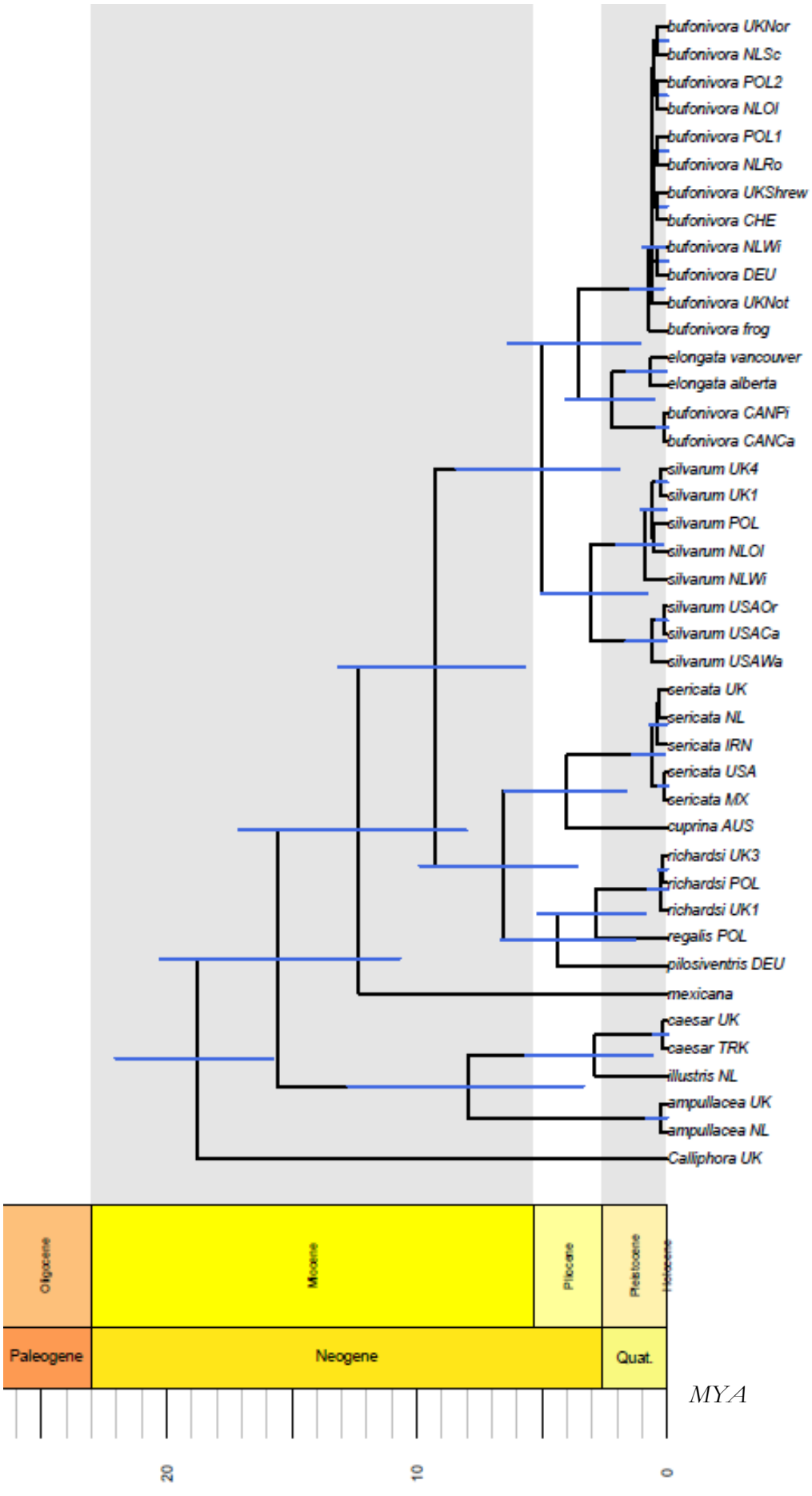
bufonivora UKNor
bufonivora NLSc
bufonivora POL2
bufonivora NLOl
bufonivora POL1
bufonivora NLRo
bufonivora UKShrew
bufonivora CHE
bufonivora NLWi
bufonivora DEU
bufonivora UKNot
bufonivora frog
elongata vancouver
elongata alberta
bufonivora CANPi
bufonivora CANCa
silvarum UK4
silvarum UK1
silvarum POL
silvarum NLOl
silvarum NLWi
silvarum USAOr
silvarum USACa
silvarum USAWa
sericata UK
sericata NL
sericata IRN
sericata USA
sericata MX
cuprina AUS
richardsi UK3
richardsi POL
richardsi UK1
regalis POL
pilosiventris DEU
mexicana
caesar UK
caesar TRK
illustris NL
ampullacea UK
ampullacea NL
Calliphora UK
Oligocene
Miocene
Pliocene
Pleistocene
Holocene
Paleogene
Neogene
Quat.
MYA
20
10
0

Figure 4.12 Divergence times estimated from a concatenated dataset of *per*, *COX1* and *ITS2* for the *Lucilia bufornivora* species group. Substitution model and relaxed clock models were unlinked to each gene. The tree was callibrated by setting the root to the node age corresponding to the split between Luciilinae and Calliphorinae subfamilies (~19 mya) estimated by Wallman et al. (2005). Blue bars represent 95% highest posterior density (HPD) of each node age. Scale represents number

4.4 Discussion

4.4.1 Phylogenetic relationships

Previous studies have suggested that the parasitic habit in *Lucilia* blowflies evolved independently multiple times (Stevens and Wall, 1997a; Stevens, 2003; Stevens and Wallman, 2006). Present results support this hypothesis, as there was a clear distinction between the different *Lucilia* species-groups, most of which include taxa that exhibit both saprophagous and parasitic life histories. However, obligate parasitism and high specialisation for a distinct host group appear to have evolved just once, as indicated by the reciprocal monophyly of *L. bufonivora* and *L. elongata*. On the other hand, although *L. silvarum* showed close relationships with the latter two species, it was never incorporated to the monophyletic group of taxa associated with obligate amphibian myiasis. This is reasonable given the fact that *L. silvarum* exhibits saprophagous feeding habits, which have been well documented in the past (Hanski and Kuusela, 1977; Hanski, 1987; Prinkkila and Hanski, 1995; Fremdt et al., 2012).

By default, mutation rates in mtDNA are faster than the those in nuclear DNA due to the lack of recombination and the accumulation of deleterious mutations (Neiman and Taylor, 2009; Brown et al., 1979). Within recently diverged species of *Lucilia* blowflies, this can result in shorter branches in nuclear phylogenies but larger in mtDNA phylogenies (McDonagh and Stevens, 2011; Yusseff-Vanegas and Agnarsson, 2017). This has also been reported in other insect groups like cabronid wasps (Hymenoptera) (Kaltenpoth et al., 2012). The rapid mtDNA substitution rates cannot be attributed to the parasitic life-style of the toad fly, as our results suggest that its saprophagous ancestor, *L. silvarum*, was already showing a fast mtDNA mutation rate.

All phylogenies inferred in this study recovered a Nearctic clade of *L. bufonivora* which is distinct from a Palearctic clade of the same species, and therefore, consistent paraphyly of

L. bufonivora with respect to *L. elongata*. Both the mtDNA phylogeny (*COXI*) and parsimony splits network (*ITS2* + *per*) suggest that *L. bufonivora* from Canada has greater affinity with the strictly Nearctic *L. elongata* than with its Palearctic conspecifics. Surprisingly, *COX1* intraspecific pairwise distance between Canadian and European individuals of *L. bufonivora* was greater than the interspecific distance displayed by the sheep blowflies *L. sericata* and *L. cuprina*. Thus, geographical isolation of *L. bufonivora* and rapid mtDNA evolution rates appear to be facilitating on-going cryptic speciation. This phenomenon is relatively common within Diptera, as reported in geographically isolated populations of gall midges, tephritid flies and black flies (Tadeo et al., 2015; Adler et al., 2016; Duque-Gamboa et al., 2018). The status of *L. bufonivora* in Canada as a distinct species, however, remains to be resolved, and will also require detailed morphological examination of specimens from both Eastern and Western hemispheres

Using nDNA (*EF1α*), the work from the previous Chapter grouped *L. bufonivora* as a separate species to *L. silvarum* and *L. elongata*. Nevertheless, BI analysis of the nuclear gene *EF1α* failed to differentiate the saprophagous *L. silvarum* to the obligate *L. elongata*, as they were grouped within the same clade. The results presented in this chapter provided better phylogenetic resolution with nDNA evidence from two markers (*ITS2* and *per*) and grouped *L. elongata* as a distinct species to *L. silvarum* and *L. bufonivora*. This is also supported by recent morphological evidence (Tantawi and Whitworth, 2014). Previous research has showed the utility of *ITS2* as a suitable marker to infer relationships at species level (Marinho et al., 2011). Certainly, *L. elongata* presented a unique consistent haplotype that allows its differentiation to *L. bufonivora* and *L. silvarum*. Therefore, unambiguous species identification can be carried out employing multi-locus analysis with *COX1* and *ITS2* sequence data.

The saprophagous species *L. silvarum* exhibited high mtDNA sequence divergence between Palearctic and Nearctic samples. Although it could be concluded that this is due to species level differentiation, it should rather be interpreted with caution. For instance, previous molecular studies on other blowflies, such as *Phormia regina* (Meigen), detected high mtDNA sequence divergence between North American and European populations (Boehme et al., 2012; Desmyter and Gosselin, 2009). Due to the lack of morphological differentiation and minimal nuclear DNA variation, it was concluded that its mtDNA variation is not a species level differentiation (Jordaens, et al., 2013). This phenomenon has also been reported for populations of *Lucilia eximia* (Wiedemann) and *Lucilia rica* (Shannon) (Yusseff-Vanegas and Agnarsson, 2017). In addition, the BI analysis from the *per* gene clustered Nearctic and Palearctic in a single clade. Although with little variation, the parsimony splits network

grouped together all samples of *L. silvarum* with low distances, which was not a feature observed between Nearctic and Palearctic *L. bufonivora* (Fig. 4.11). Therefore, mtDNA variation suggests independent evolution rates of two isolated populations of *L. silvarum* but cannot be attributed to species level differentiation.

Evidence from mtDNA (*COX1*) revealed that *L. thatuna*, another species that has been thought to be involved in amphibian myiasis (Tantawi and Whitworth, 2014), does not have close relationships with the *L. bufonivora* species-group. Given the fact that all inferred phylogenies support that evolution of specialisation for amphibians occurred only once, it is likely that *L. thatuna* does not behave as such. This matches with previous phylogenetic studies that have found no close relationships between *L. thatuna* and *L. silvarum*/*L. elongata* (DeBry et al., 2010; Debry et al., 2013). Thus, suggesting *L. thatuna* might only exhibit saprophagous feeding habits.

The present results revealed that the rarely encountered species *L. pilosiventris* and *L. regalis* have close relationships with *L. richardsi*, conforming a species group which is related to both *L. sericata* and *L silvarum*, hence suggesting saprophagous behaviour. Little is known about their biology and life-history due to their low abundance (Rognes, 1991; Szpila, 2017). In fact, there is only one morphological-based phylogenetic study that has included these species (Stevens and Wall, 1996) although this was based on species descriptions given in the literature. The latter study found that *L. pilosiventris* and *L. regalis* are related to *L. silvarum*, which was also supported by the present mtDNA phylogeny (Fig. 4.9). This, however, is incongruent with the *ITS2* phylogeny, as they seem to be more closely related to the *L. sericata* species group. In *Drosophila* flies, these incongruencies are attributed to incomplete lineage sorting (Pollard et al., 2006). The incongruencies observed in mtDNA and nDNA phylogenies from this study might also be a case of incomplete lineage sorting. For instance, the toad fly species group (*L. bufonivora*/*L. silvarum*/*L. elongata*) and the sheep blowfly species group (*L. sericata*/*L. richardsi*/*L. regalis*) seem to have a common ancestor (Figs. 4.7 – 4.12). It is likely that after the rapid speciation of this ancestral state, polymorphisms were fixed randomly in each species (e.g. *L. sericata* and *L. bufonivora*), and in some cases of non-sister species it would result on the fixation of the same ancestral polymorphisms (e.g. *L. richardsi* and *L. silvarum*). Nonetheless, our studies are limited to a single mtDNA locus, and further studies with more loci or mitogenomic data are required to confirm this hypothesis.

4.4.2 Evolution of obligate parasitism in *Lucilia* blowflies and host specificity for amphibians

It is thought that economically important calliphorid flies (i.e. *Lucilia sericata*, *L. cuprina*) might have evolved parasitic behaviour in association with humans and animal domestication, as myiasis is rarely seen in wild animals (Erzinclioglu, 1989; Stevens and Wall, 1997). However, high host-specificity for wild amphibians suggests that *L. bufonivora* evolved independently from those blowfly species associated with animal domestication.. Indeed, the time-scaled phylogeny suggests that this behaviour arose approximately 5mya, during the early Pliocene (~5 mya). In some groups of strictly obligate taxa such as Oestrid flies, host-parasite coevolution could have played an important role on lineage divergence and speciation (Pape, 2006; Stevens et al., 2006). This, however, differs largely to the evolution of *L. bufonivora* as it shows close affinity with other fly species with predominantly saprophagous feeding habits (*L. silvarum*).

Present results indicate that the most recent ancestor of *L. bufonivora* exhibited saprophagous feeding habits. Certainly, all phylogenies suggest reciprocal monophyly of the toad fly (*L. bufonivora*) and the sheep blowfly (*L. sericata*) species groups; both comprised by parasite and saprophagous taxa (Fig. 4.7, Fig. 4.9, Fig. 4.10). The time-scaled phylogeny suggests the co-existence of the saprophagous ancestor of *L. bufonivora* with other Calliphorid lineages that behave mostly as carrion-breeders (e.g. *Calliphora*). It is well known that ephemeral resources such as it is carrion, facilitate intense interspecific competition (Hanski and Kuusela, 1977; Hanski, 1987; Prinkkila and Hanski, 1995). It is also thought that *L. silvarum*, a closely related species to *L. bufonivora,* is a very poor competitor of the carrion-fly community (Hanski, 1987). Thus, intense competition within the carrion-fly community might have forced the saprophagous ancestor of *L. bufonivora* to migrate to narrower ecological niches. In this case developing high host-specificity for amphibians, facilitating and effective adaptative radiation of an evolutionary lineage of obligate parasites, namely *L. bufonivora*.

As previously discussed, geographical isolation facilitated the divergence of this evolutionary lineage, resulting in the speciation of *L. bufonivora sensu stricto* in the Palearctic and a distinct *L. elongata* + *L. bufonivora* in the Nearctic. The estimates from this work suggest that this split took place around the Pliocene (~3.5mya, Fig. 4.12). Given the difference of amphibian diversity, they have adapted to different hosts according to the geographical area. For instance, in Europe amphibian myiasis is normally reported occurring in the nasal nostrils of the common toad, *Bufo bufo*. In North America, however, it is reported from the back and

hind legs of mainly frog hosts (Zumpt, 1965; Strijbosch, 1980; Roberts, 1998; Eaton et al., 2008).

Despite the ectoparasitic behaviour of the sheep blowfly *L. sericata,* it typically behaves as a saprophagous species in a wide range of countries, and in contrast with *L. bufonivora*, it is a very common blowfly species (Hwang and Turner, 2006; Saloña-Bordas et al., 2009; Fremdt and Amendt, 2014; Lutz, 2019) . Human migrations and movement of livestock could have played an important role on the intercontinental dispersal of economically important calliphorid species, such as the new world screwworm *C. hominivorax* (Fresia, et al., 2013). Thus, movement of domesticated sheep might have had great implications on the distribution of *L. sericata*, and moreover, its saprophagous feeding habits could have facilitated its establishment in different geographical areas. Big population sizes, high migration capacity and fertility increase the rates of gene flow and reduce the impact of genetic drift over this species (Diakova, et al., 2018). This would explain the genetic consistency of geographically distant samples of *L. sericata* found in this study, which matches largely with previous research that has found very minimal variation in *L. sericata* (Stevens and Wall, 1997b; DeBry et al., 2010; McDonagh and Stevens, 2011; Williams and Villet, 2013). In contrast, the low abundance of *L. bufonivora* in the field suggest that small population sizes, in combination with a restricted dispersal capacity, make the toad fly a vulnerable species to genetic drift, therefore, facilitating the rapid evolution of geographically isolated populations.

Given that the toad fly, *L. bufonivora*, parasitizes mainly wild hosts, it is unlikely that human activity mediated its intercontinental dispersal. There is not enough robust evidence to conclude how it migrated between continents. Nonetheless, the present time-scaled phylogeny suggests that it occurred during the Pliocene, which was a determining epoch for intercontinental dispersal of vertebrates, such as mammals, through Beringia (Cook et al., 2017). The latter is also known to have mediated intercontinental dispersal of plants, amphibians and even insects (Wen et al., 2016; Contreras and Chapco, 2006; Cook et al., 2017; Li et al., 2015). Although there are existing reports of *L. bufonivora* from far east Asia and North Canada (Draber-Mońko, 2013; Tantawi and Whitworth, 2014), more detailed phylogeographic studies as well as updated surveys on the Calliphorid fauna from Eastern Russia and Alaska are required to answer this question. Nevertheless, it can be concluded that *L. bufonivora* has been present in the North American continent for at least 2 million years but has remained unrecorded due to its relative rarity as well as taxonomic confusion with *L. silvarum*.

4.4.3 Species composition in Amphibian myiasis in North America.

There are numerous reports of the saprophagous *L. silvarum* causing amphibian myiasis in North America (Bolek and Coggins, 2002; Bolek and Janovy, 2004; Roberts, 1998). Although, as in Europe, the present study suggests that those cases are likely to be attributed to *L. bufonivora*. For instance, the morphological keys widely used for fly identification in North America (Hall, 1948) do not even include the taxon *L. bufonivora*. It was not until 2014 that Tantawi and Whitworth (2014) provided morphological keys for its accurate identification and differentiation with *L. silvarum*. Moreover, the latter study found *L. bufonivora* specimens misidentified as '*L. silvarum*' in Canadian collections with 1954 as the earliest collection record. Certainly, present DNA analysis of the Canadian samples originally provided as '*L. silvarum*' (reared from diseased amphibians) revealed their identity as *L. bufonivora*. Therefore, this is the first study to confirm its involvement in amphibian myiasis in Alberta, Canada. Nonetheless, more detailed studies are required to determine the amphibian myiasis species composition in North America.

In conclusion, within the genus *Lucilia,* obligate parasitism and host-specificity for amphibians is likely to have evolved just once around 4 mya. It is likely that this occurred after the niche displacement of a generalist saprophagous ancestor from carrion-fly community. Consistent paraphyly of *L. bufonivora* across single-gene phylogenies and high mtDNA sequence divergence between Palearctic and Nearctic lineages suggest on-going cryptic speciation of *L. bufonivora* facilitated by geographical isolation. The time-scaled phylogeny suggests it has been evolving independently in these 2 regions for at least 2mya. Thus, this species appears to have been present in North America since this time, but due to its relative rarity it has remained unrecorded by taxonomists until relatively recently (Tantawi and Whitworth, 2014).

5. Abundance, bait response and habitat use by adult flies of *Lucilia bufonivora*

Contributions

Gerardo Arias-Robledo was the main researcher and undertook all sample collection and identification as well as statistical analysis and result interpretation. Richard Wall assisted as the main supervisor and provided advice on statistical methods. Jamie Stevens supported with advice on data sampling as well as organising the field work season. Tariq Stark offered guidance on the location of trapping sites. Annemarieke Spitzen provided toad tissue for data sampling as well as access to a sampling site. Kris Joosten, Bas Budel and Anne Buitenhek provided access to sampling sites. The Veterinary Parasitology and Ecology group provided consumables for fieldwork. The work was funded by CONACyT and University of Bristol

5.1 Introduction

The toad fly, *Lucilia bufonivora*, is a species that has remained understudied for several reasons. For instance, given the nature of its obligate form of parasitism, it does not provide ecosystem services, as most saprophagous calliphorids do as decomposers (Putman, 1983; Blackith and Blackith, 1990; Smith and Wall, 1997b). This also means that it is not an important species in forensic entomology (Fremdt and Amendt, 2014). Moreover, while economically important species of *Lucilia* (e.g. *Lucilia sericata* and *Lucilia cuprina*) are facultative parasites of domesticated animals (Zumpt, 1965; Wall et al., 1992a; Stevens, 2003), the toad fly affects mostly wild amphibian hosts (Brumpt, 1934; Strijbosch, 1980; Weddeling and Kordges, 2008; Gosá et al., 2009) and, hence does not have the same economic importance.

Nonetheless as previously discussed in previous chapters, the study of this species is of interest in evolutionary entomology, due to its life-history as an obligate parasite amongst species with sarco-saprophagous habits (McDonagh and Stevens, 2011). Understanding the diversity of host-parasite relationships is important for conservation, hence it is also of importance for wildlife management (Hatcher et al., 2006; Hatcher et al., 2012). Additionally, *L. bufonivora* has been reported in a range of amphibian hosts, including a species which is currently endangered in the U.K., the Natterjack toad, *Epidalea calamita* (Vestjens, 1958; Weddeling and Kordges, 2008; Gosá, et al., 2009).

It is known that parasites and pathogens play important roles within a community and can affect the complexity of food-webs and energy budgets of an ecosystem (Hatcher et al., 2006; Hatcher et al., 2012). Research on *L. bufonivora* has been focused typically on the study of the immature stage, as it is responsible for amphibian myiasis (Brumpt, 1934; Strijbosch, 1980). A study from Germany found infestation rates in toad populations from 15% and 70% (Weddeling and Kordges, 2008). In the Netherlands, *L. bufonivora* is more frequent in adult toads of *Bufo bufo*, with an average of 8% of individuals reported to be infected in a two-year period (Strijbosch, 1980). However, the ecological impact of *L. bufonivora* on amphibian populations, remains poorly understood. Part of this is due to the lack of knowledge on the ecology and abundance of the adult stage of this species. A broader understanding of the behaviour of the adult stage of the parasite is important for a number of reasons. Firstly, dispersal and reproduction of most dipterous parasites is carried out during their adult stage (Zumpt, 1956; Zumpt, 1965; Wall, 1992; Pape, 2001). Thus, ecological studies can provide valuable data to assist on further prediction of the spaces where the hosts (if any) are more

vulnerable to oviposition/infection. Secondly, it would offer knowledge on a calliphorid fly of which behaviour remains poorly studied (Zumpt, 1965; Rognes, 1991). Investigation of its ecology (e.g. spatial distribution) would also help understanding sympatry and co-existence with respect to other calliphorid species and assist on the prevention of future misidentifications and erroneous biodiversity reports, which are known to happen commonly (Rognes, 2014; Tantawi and Whitworth, 2014).

Previous research has shown that the type of bait influences the species composition in blowfly trapping (MacLeod and Donnelly, 1956; Blackith and Blackith, 1990). As discussed in Chapter 2, females of saprophagous blowflies are attracted to carrion and decaying meat as they use this source for larvae development (Smith and Wall, 1997b; Fisher et al., 1998). Additionally, it also attracts newly emerged adult females that are looking to obtain a proteinaceous meal that enables vitellogenesis prior to oviposition (Fisher et al., 1998; Huntington and Higley, 2010). In contrast, as an obligate parasite, *L. bufonivora* needs a live host for oviposition and larvae development (Brumpt, 1934; Zumpt, 1965). This has possibly been reflected in the low abundancies reported by previous authors that used standard blowfly baits (MacLeod and Donnelly, 1956; Fischer, 2000). Nonetheless, it has been suggested that adults are attracted to dead toads for 'feeding' (Zumpt, 1965). This, however, remains to be confirmed.

Relatively little is known about habitat use and the spatial distribution of *L. bufonivora*. Macleod and Donnelly (1956) reported low catches of *L. bufonivora* in the UK, and they did not describe the type of habitat in in which they did catch it. However, the same authors noted that its sister species *Lucilia silvarum* was more frequently caught in non-shaded habitats. Fischer (2000) found that adult *L. bufonivora* were more abundant in forests and along riverbanks. In contrast, amphibian myiasis cases are frequently reported from sunnier environments. In the Iberian peninsula, several cases of amphibian myiasis have been recovered from meadows and wetlands from different Natural Reserves (Gosá et al., 2009). Weddeling and Kordges (2008) observed that infested amphibians occur more frequently in open landscapes or beside ponds but rarely in shaded forests. In the Netherlands, a study recovered toad-myiasis cases from different habitats including heaths, pastures, meadows and farmyards (Strijbosch, 1980).

The work described in this chapter was a collaboration with RAVON (Reptile, Amphibian and Fish Conservation in the Netherlands). It aimed to investigate the spatial distribution, bait response and abundance of adult *L. bufonivora* in three different sites of the Netherlands at which, according to the RAVON database, toad myiasis cases had been reported in the past. Additionally, this work also aimed to provide data on the general *Lucilia*-species community from the Netherlands.

5.2 Methods

5.2.1 Study sites

Site 1

Site one was on a small farm situated in a rural area situated in Wesepe, Olst (Fig. 5.1). It is surrounded by corn fields; small woodland patches with mainly birch and oak trees; and relatively large open and semi-open wetland and grassland with Apiaceae vegetation, (Fig. 5.2). Amphibians reported at the site included the common toad (*B. bufo*) and the common frog (*Rana temporaria*).

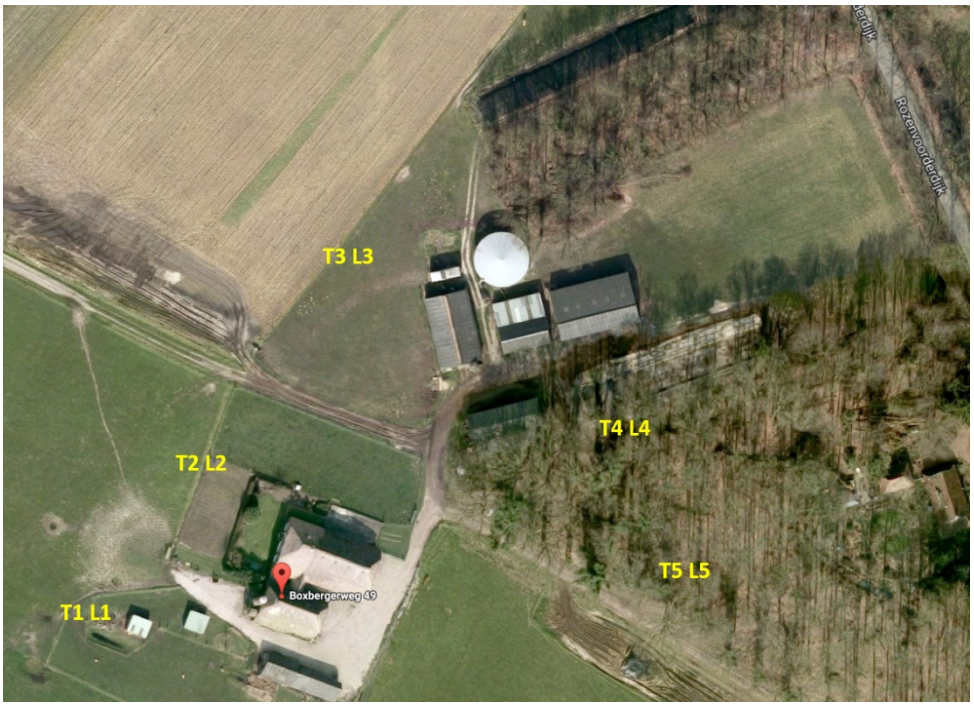

Figure. 5.1. Sampling site 1 (Olst). Trap number and bait (L=liver, T=Toad) are shown in the picture. Six traps were set at open or semi-open habitats (T1-T3; L1-L3) and four traps at woodland shaded habitats (T4, T5, L4 and L5).

Figure. 5.2. Dominant habitats at site 1 were mainly: a) open spaces with abundant Apiaceae and grassland vegetation and b) grassland surrounded by cropfields.

Site 2

Stie 2 was located in a semi-rural area in Winssen, approximately 10 miles away from the town of Winssen (Fig. 5.3). The site was a private garden situated approximately half a mile away from the river Waal. It is surrounded by cattle farms, arable land, woodland patches (Oak, birch and willow) and a small semi-natural grassland with small native fruit trees and abundant apiaceae vegetation (Fig. 5.4). Amphibians reported at the site included the common toad (*B. bufo*), the common frog (*R. temporaria*), the edible frog (*Pelophylax esculentus* Linnaeus) and the common newt (*Lissotriton vulgaris* Bell).

Figure. 5.3. Sampling site 2 (Winssen). Trap number and bait (L=liver, T=Toad) are shown in the picture. Four traps were set at open or semi-open habitats (T4, T5, L4 and L5) and six traps at woodland and shaded habitats (T1-T3; L1-L3).

Figure. 5.4. Habitats surveyed at site 2. Traps were set at woodland habitats that provided shade (a– b) or at semi-open habitats that provided direct or partial sunlight (c).

Site 3

Site three was within the botanical and experimental gardens of Radboud University (Fig. 5.5). Situated in an urban area with dense housing in central Nijmegen. It is mainly composed by forest vegetation with a variety of native and non-native trees, pines and ferns (Fig. 5.6). It has a pond lying on the middle of the garden. Within site, trapping access was restricted only to woodland areas, therefore grassland habitats were not surveyed. Amphibians reported at the site include the common toad (*B. bufo*), the common frog (*R. temporaria*), the edible frog (*P. esculentus.*), common newt (*L. vulgaris*) and the alpine newt (*Ichthyosaura alpestris* Laurenti).

Figure 5.5. Sampling site 3 (Botanical Gardens - Nijmegen). Trap number and bait (L=liver, T=Toad) are shown in the picture. Only woodland habitats were surveyed from this site.

Figure 5.6. Dominant habitat at site 3 were mainly shaded woodland spaces with a variety of tall trees and fern vegetation that offered shade to the traps (a -b). Modified traps used for blowfly sampling were attached to the stems of tall vegetation present in woodland (c).

5.2.2 Bait response

To measure the differences in the abundance of *L. bufonivora* using different baits, porcine liver (standard blowfly-bait) or toad carcasses were used for fly sampling. The former was obtained from a local butcher in Nijmegen, while toad carcasses (death by natural causes) were provided by A. Spitzen (RAVON). Modified bottle traps (Hwang and Turner, 2006) were used to catch specimens in sufficiently good condition to allow identification. Fifty grams of bait (liver or toad) were placed in a plastic container and it was covered with a mesh and a rubber band to reduce oviposition. The container was then placed inside the trap. Throughout the trapping period, baits were topped up with water to prevent their desiccation. It has been suggested that after the initial stages of decomposition, bait age has little effect over blowfly catch size (Fisher et al., 1998) so bait age was not standardised. As the experiment did not last more than 2 months and due to bait availability (toad carcasses), baits were not replaced (unless removed by external factors, such as strong winds, scavengers, etc.).

5.2.3 Effects of Habitat

This study considered 2 different types of habitats for fly sampling. The first one was shaded areas, mainly woodland and forests which offered wind cover and no direct sunlight to the traps (Fig. 5.4 a-b; Fig. 5.6). Within these areas, traps were set-up by attaching them to tree stems present within the area. The second habitat was open or partially open landscapes, typically with low vegetation such as grass or Apiaceae, that allowed direct sunlight to the traps (Fig. 5.2; Fig. 5.4c). Traps were attached to pre-existing fence-posts or bushes in these open areas (Fig. 5.4c).

5.2.4 Trap distribution, collection and identification

Traps were placed in pairs: one liver-baited and one toad-baited, separated by approx. 6-8m. The distance between pair of traps was approximately 30 meters. Numbers of traps at each site was determined by the relative amount of each habitat-type available. At site 1 (Olst), 6 traps were set in open and semi-open habitats and 4 traps in woodland (Fig. 5.1). In site 2 (Winssen), 4 traps were set in semi-open areas and 6 were set in shaded habitats (Fig. 5.2).

Finally, given that site 3 (Nijmegen) was mainly woodland, fly-sampling in this site was only carried out only in shaded areas (Fig. 5.3).

At site 1, traps were set on the 10th of August, 04th of August at site 2, and 9th of August at site 3. Collections were made every three to six days for four weeks from the day the traps were set. At every collection, the upper part of the bottle traps was emptied in individual collecting containers. Each container was labelled with its respective collection date, site, bait and habitat sampled. Traps were then placed back in their respective place in the field. Flies were then frozen and morphological identification carried out at the University of Bristol during late-September and October 2017.

Containers were emptied and green iridescent flies were separated from non-target species for identification. Morphological keys were employed for reliable morphological ID (Rognes, 1991). Although indicated in several keys, this work did not consider post-acr bristles as a differentiation character between *L. bufonivora* and *L. silvarum* as it is not consistent between individuals. Instead, genitalia were examined under a dissecting microscope, in case of females *L. bufonivora* was identified by the presence of microtrichia on the epiproct and the abdominal terguite and sternite 7 (Fig. 5.7 – Fig. 5.8). The number of specimens per trap at each collection was recorded for individual species of *Lucilia*.

5.2.5 Data analysis

This work considered the spatial distribution and abundance of *L. bufonivora*. Given that fly-sampling lasted less than 2 months, it was not possible to analyse temporal changes in abundance. Hence, analysis was carried out using the number of flies/trap/day for individual species of *Lucilia* as described in chapter two. Count data for all *Lucilia* studied exhibited overdispersion (Fig 5.9). Thus, a generalised linear mixed model was used for data analysis of individual species. The best-fit model was selected using appropriate model families for overdispersed data and stepwise removal of non-significant factors was undertaken based on AIC scores (Sileshi, 2006). Effects of bait-type and habitat on the abundance of individual *Lucilia* species were evaluated as fixed factors. Due to the difference on the trapping starting date and inconsistency in surveyed habitats per site, 'site' was implemented as a random factor using the package Lme4 (Bates et al., 2015) with the function glmer.nb using R in RSTUDIO 3.4.2. To test whether 'site' had a significant effect on the fly catch, the selected model and a null model (without site) were subjected to ANOVA.

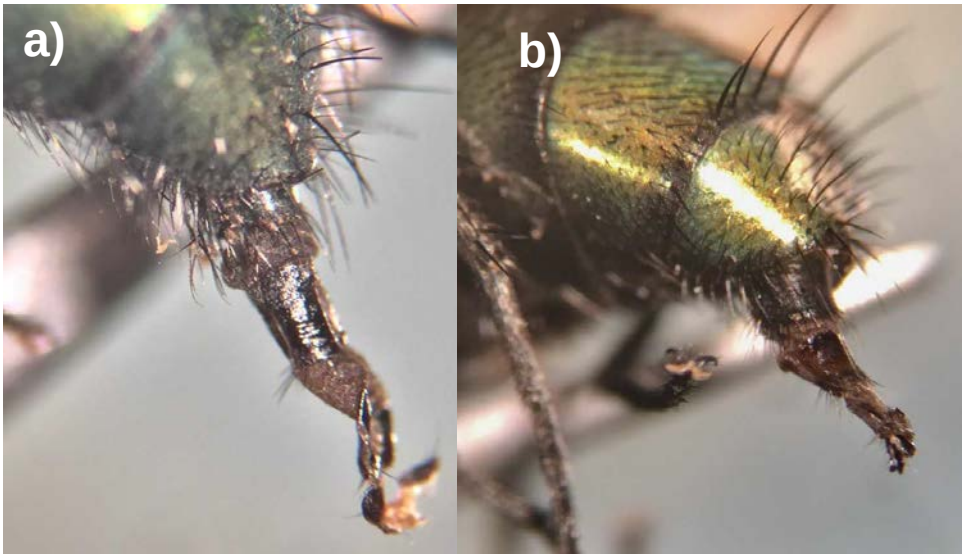

Figure 5.7. Ovipositor of: a) *Lucilia silvarum* and b) *Lucilia bufonivora*. Pictures taken by Abby Parravani (University of Bristol).

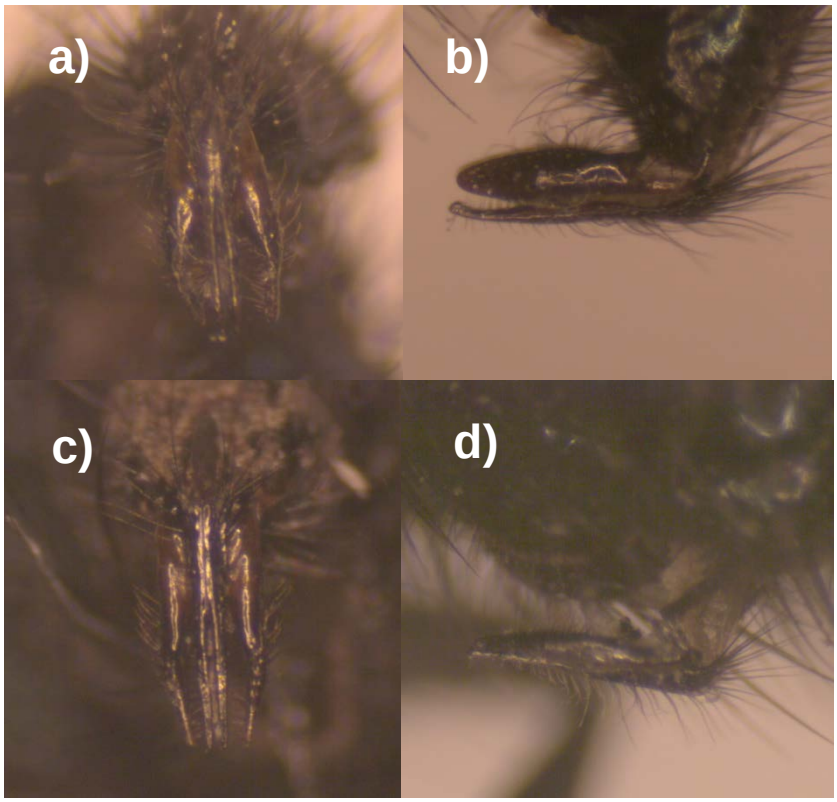

Figure 5.8. Male terminalia of *Lucilia bufonivora*: a) ventral view and b) dorsal view. Male terminalia of *Lucilia silvarum*: ventral view (c) and dorsal view (d).

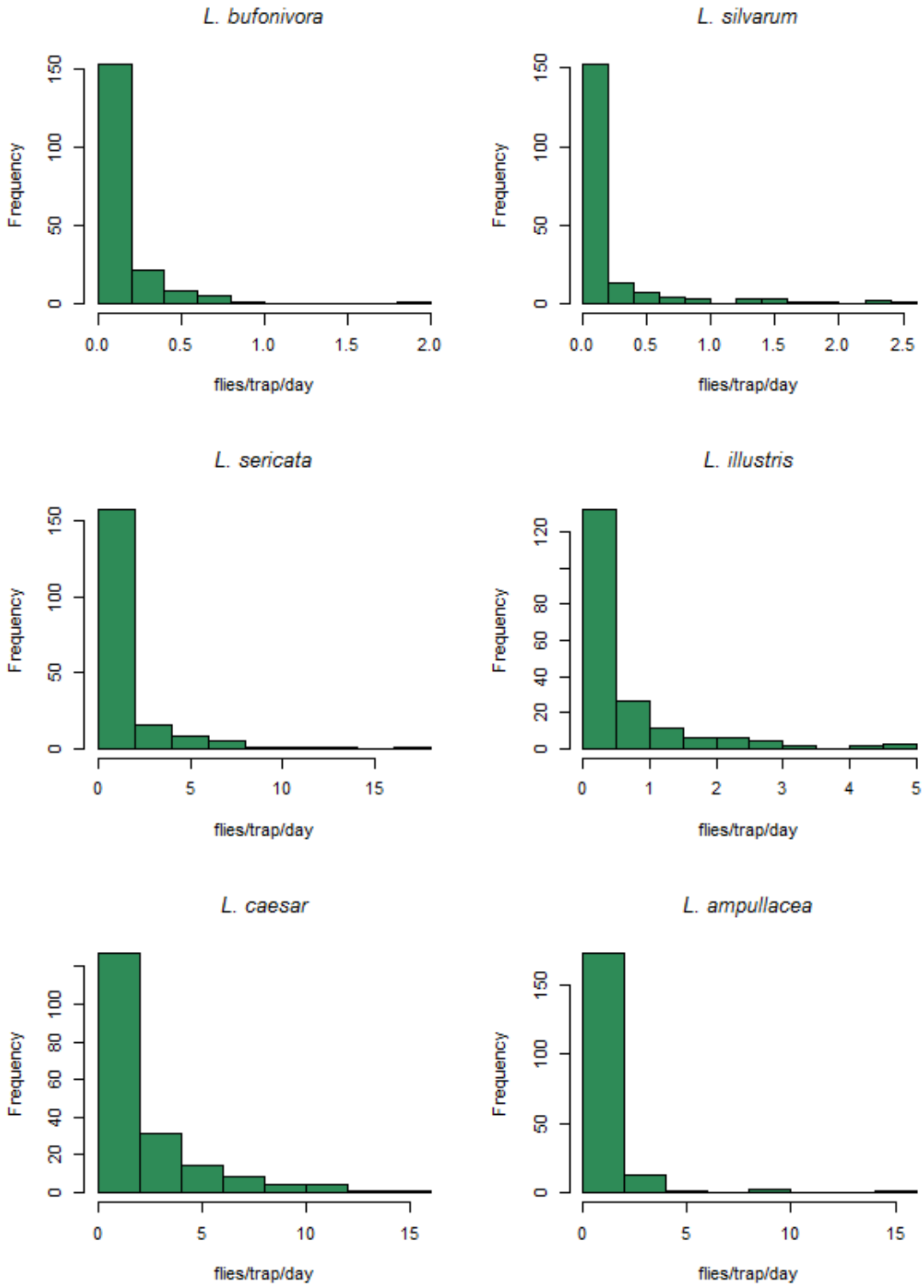

Figure 5.9. Distribution frequencies of the *Lucilia* species recorded in this study. *X* axis displays the flies/trap/day and *Y* axis represents the frequency. Individual species names are indicated at the top of their respective histogram plot.

5.3 Results

A total of 3,855 *Lucilia* flies were collected and six different species were identified in this study (Table 5.11); 77 individuals were *L. bufonivora*, 133 *L. silvarum*, 959 *L. sericata*, 381 *L. illustris*, 579 *L. ampullacea* and 1,756 *L. caesar*.

5.3.1 Model selection

Lucilia bufonivora

Any model within **Δ**AIC=2 are considered equally likely, in these cases it is generally suggested that the simplest model is accepted. In this analysis, three models fell within this rule (Table 5.1). From these, the simplest model included 'habitat' as a fixed factor and 'site' as a random factor and excluded the fixed effects of 'bait' from the analysis. This suggests that 'bait' has no appreciable effect on the catch of *L. bufonivora*. In contrast, 'habitat' was a significant factor affecting the numbers of *L. bufonivora* caught ($Z=-5.31$, $P<0.001$); higher numbers were caught in open habitats and it was almost absent from shaded areas (Fig. 5.10). ANOVA using the factors included in the optimum and null models showed that 'site' had a significant effect on the fly catch size of *L. bufonivora* ($\chi2(1) = 8.65$, $P<0.001$). No specimens were caught at Site 3, and the highest abundance recorded was at site 1.

Table 5.1. AIC scores of the different models tested for data analysis of *Lucilia bufonivora*. Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	Family	df	AIC
Buf ~ habitat	Negative binomial	3	219.381
Buf ~ bait	Negative binomial	3	316.612
Buf ~ habitat+bait	Negative binomial	4	218.569
Buf ~ habitat+(1 | site)	Negative binomial	4	212.733
Buf ~ bait*habitat*(1 | site)	Negative binomial	6	212.246
Buf ~ bait*habitat+(1 | site)	Negative binomial	5	211.233
Buf ~ bait+(1 | site)	Negative binomial	4	270.763

Letters in blue indicate the model selected for data analysis. 'Buf' represents the numbers of *Lucilia bufonivora* flies per trap per collection. '(1 | site)' indicates the inclusion of 'site' as a random factor in the given formula.

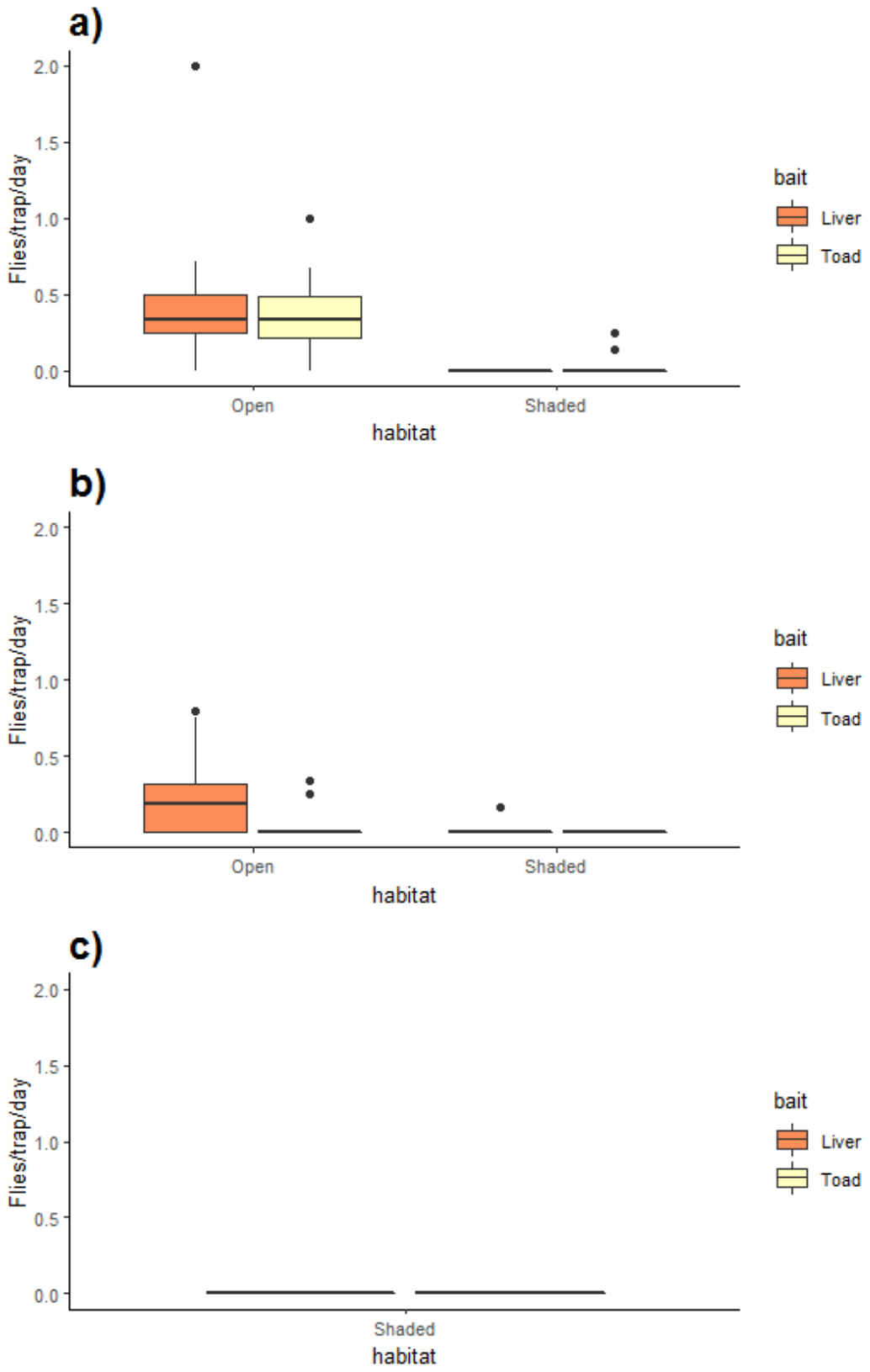

Figure 5.10. The number of *Lucilia bufonivora* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

Lucilia silvarum

The model selected for the analysis of *L. silvarum* included 'habitat' as a fixed factor and random effects of 'site' (Table 5.2). ANOVA of the selected null model showed that 'site' had a significant effect on the fly catch size of this species ($\chi2(1) = 21.38$, $P<0.0001$). It had its highest abundance at site 1 and was almost absent from site 3 (Fig. 5.11). Although the catch of *L. silvarum* was numerically greater in liver-baited traps, statistical analysis showed that it was not significantly different from toad-baited traps. Therefore, it was removed from the model resulting in lower AIC scores (Table 5.2). In contrast, 'habitat' had a significant effect on its abundance. This species was also more frequent in non-shaded open habitats (Fig. 5.11).

Table 5.2. AIC scores of the different models tested for data analysis of *Lucilia silvarum*. Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	family	df	AIC
Sil ~ habitat	Negative binomial	3	319.285
Sil ~ bait	Negative binomial	3	384.613
Sil ~ habitat+bait	Negative binomial	4	313.722
Sil ~ habitat+(1 | site)	Negative binomial	4	299.902
Sil ~ bait*habitat+(1 | site)	Corrected Poisson	5	313.073
Sil ~ bait+habitat+(1 | site)	Corrected Poisson	4	320.928
Sil ~ bait+(1 | site)	Negative binomial	4	335.447

Letters in blue indicate the model selected for data analysis. 'Sil' represents the numbers of *Lucilia silvarum* flies per trap per collection. '(1 | site)' indicates the inclusion of 'site' as a random factor in the given formula.

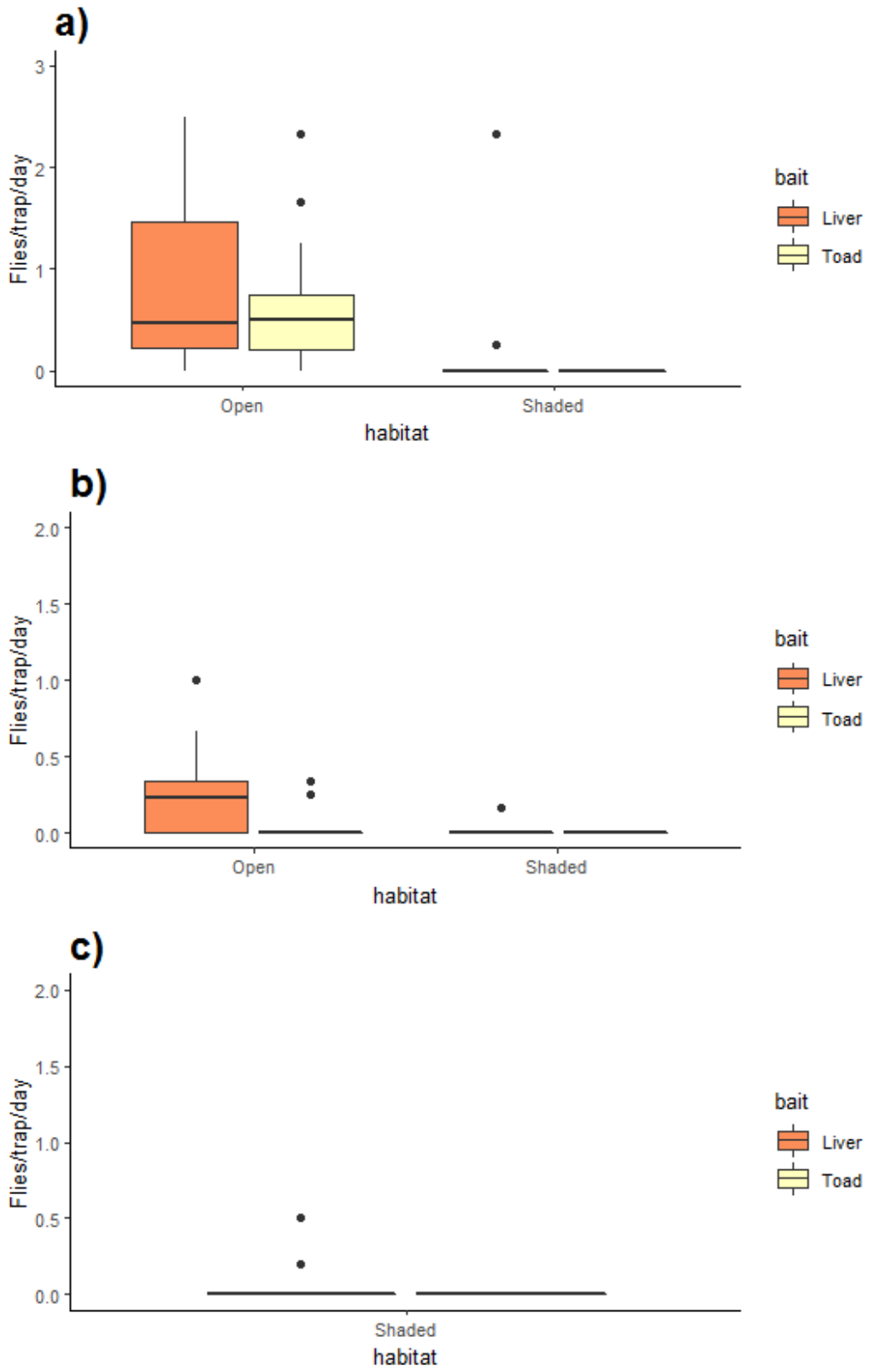

Figure 5.11. The number of *Lucilia silvarum* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

Lucilia sericata

Models with fixed effects of 'habitat' and random effects of 'site' gave a singular fit of the covariance matrix indicating that at least one variable was expressed as an exact linear combination of some of the others. An ANOVA of two null models showed that 'habitat' and 'site' were causing the singularity of the matrix, as they were not significantly different ($\chi 2(1) = -23.4$, P=1). Therefore, the random effects of 'site' were removed from the analysis. The best-fit model then included the fixed effects of both 'bait' and 'habitat', as well as the interactions between them (Table 5.3). The factor 'habitat' had a significant effect on the fly catch of *L. sericata*, as it was more abundant in open areas (Table 5.4, Fig. 5.2)

Table 5.3. AIC scores of the different models tested for data analysis of *Lucilia sericata*. Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	family	Df	AIC
Ser ~ habitat	Negative binomial	3	876.14
Ser ~ bait	Negative binomial	4	921.708
Ser ~ habitat+bait	Negative binomial	3	865.179
Ser ~ habitat*bait	Negative binomial	5	861.997
Ser ~ bait +(1|site)	Negative binomial	4	904.296
Ser ~ bait*(1|site)	Negative binomial	4	904.2959

Letters in blue indicate the model selected for data analysis. 'Ser' represents the numbers of *Lucilia sericata* flies per trap per collection. '(1|site)' indicates the inclusion of 'site' as a random factor in the given formula.

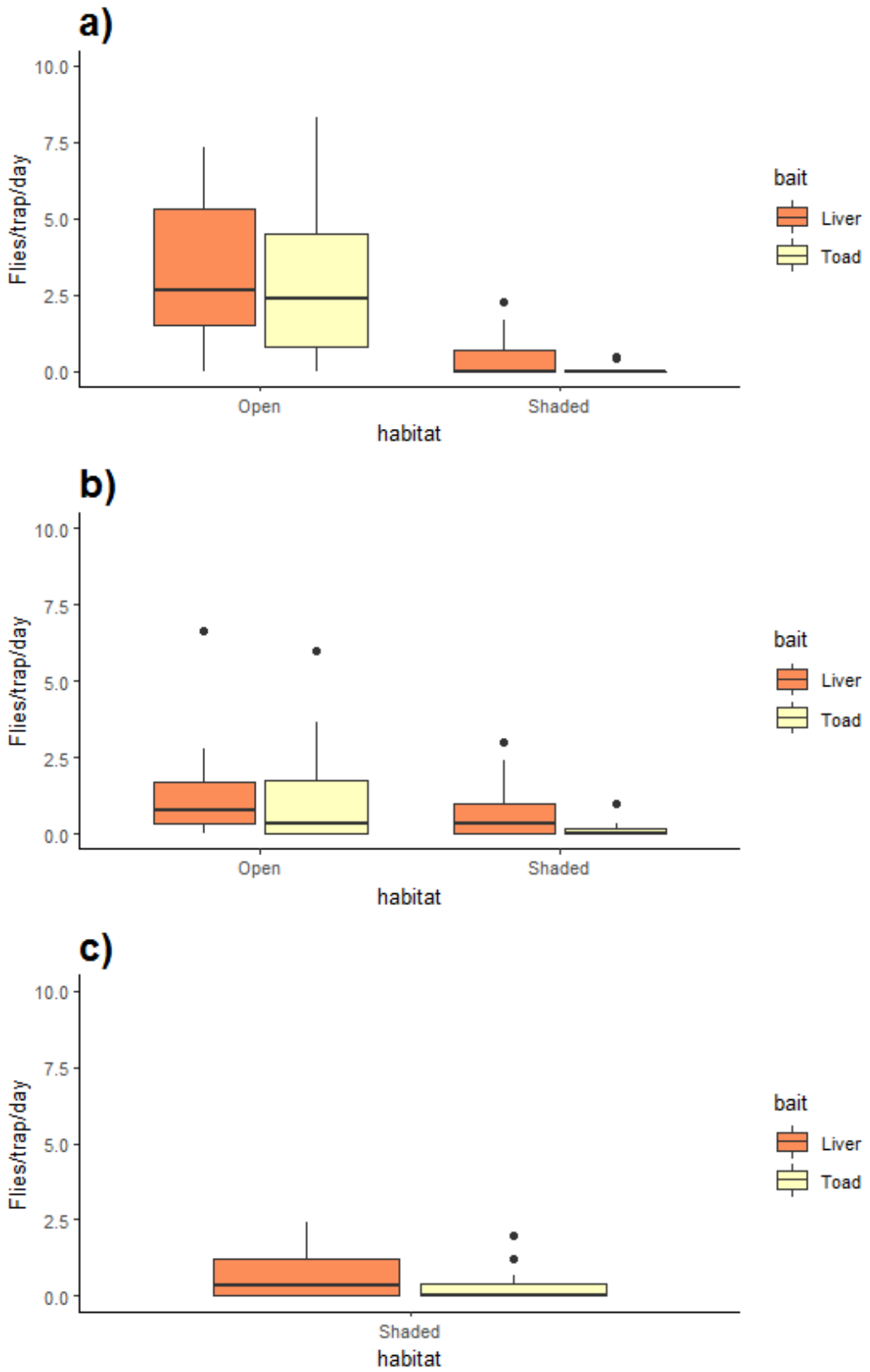

Figure 5.12. The number of *Lucilia sericata* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

Although 'bait' on its own had no significant effect on the abundance of *L. sericata*, statistical analysis showed that its interactions with 'habitat' had a significant effect on catch (Table 5.4). This indicates that in woodland areas, *L. sericata* was more frequent in liver-baited traps than it was in toad-baited traps (Fig. 5.12) but this was not the case in open habitats. This species was found in all three sampling sites (Fig 5.12)

Table 5.4. Effects of 'habitat', 'bait' and their interactions on the number of *Lucilia sericata* caught. The table displays the estimates, standard errors, z values an p values computed by the selected model.

Effects	Estimate	Std. Error	z value	p
Habitat(woods)	-1.474	0.3225	-4.574	<0.001
Bait(toad)	-0.220	0.3646	-0.605	0.545
Habitat(woods):bait(toad)	-1.091	0.473	-2.309	0.020

Lucilia illustris

Although 2 models were well within **Δ**AIC=2, the simplest one included the fixed effects of both 'habitat' and 'bait', as well as random effects of 'site' (Table 5.5). ANOVA of the selected model and a null model demonstrated that 'site' had a significant effect over the fly catch of *L. illustris* ($\chi 2(1) = 38.79$, $P<0.001$). This species was present at all 3 sampling sites, however its abundance at site 3 was the lowest reported (Fig. 5.13). Although this species was found in every habitat sampled, it was significantly more abundant in open habitats (Table 5.5). Traps baited with liver caught significantly more flies than the ones baited with toad tissue (Table 5.6, Fig. 5.13).

Table 5.5. AIC scores of the different models tested for data analysis of *Lucilia illustris*. Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	family	df	AIC
Ill ~ habitat	Negative binomial	3	638.48
Ill ~ bait	Negative binomial	3	671.624
Ill ~ habitat+bait	Negative binomial	4	633.949
Ill ~ habitat+(1 | site)	Negative binomial	4	603.147
Ill ~ bait*habitat +(1 | site)	Negative binomial	6	599.122
Ill ~ bait+habitat+(1 | site)	Negative binomial	5	597.154
Ill ~ bait+(1 | site)	Negative binomial	4	610.557

Letters in blue indicate the model selected for data analysis. 'Ill' represents the numbers of *Lucilia illustris* flies per trap per collection. '(1 | site)' indicates the inclusion of 'site' as a random factor in the given formula.

Table 5.6. Effects of 'habitat', 'bait' on the number of *Lucilia illustris* caught. The table displays the estimates, standard errors, z values an p values computed by the selected model.

Effects	Estimate	Std. Error	z value	p
Habitat(woods)	-0.97	0.237	-4.097	<0.001
Bait(toad)	-0.66	0.227	-2.885	0.004

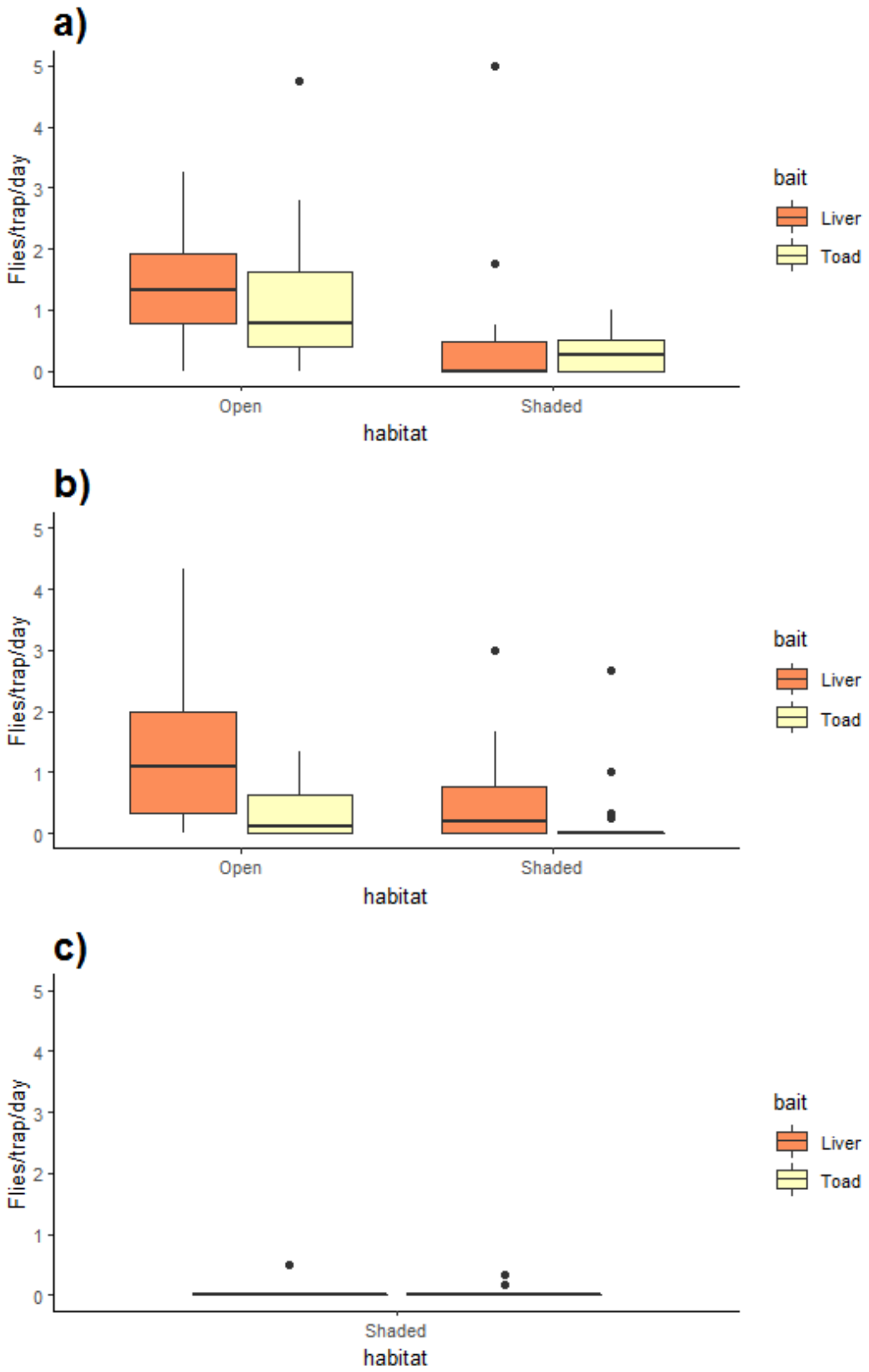

Figure 5.13. The number of *Lucilia illustris* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

Lucilia caesar

Model selected included 'habitat' and 'bait' as fixed factors as well as random effects of 'site' (Table 5.7). The latter factor had a significant effect over the fly catch size of *L. caesar* ($\chi 2(1) = 18.508$, $p<0.001$). *L. caesar* was recovered from all 3 sites (Fig. 5.14). It was significantly more abundant in woodland habitats (Table 5.8, Fig. 5.14). Liver-baited traps caught significantly more adult flies than toad-baited traps (Table 5.8, Fig. 5.14).

Table 5.7. AIC scores of the different models tested for data analysis of *Lucilia caesar*. Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	family	df	AIC
Cae ~ habitat	Negative binomial	3	1169.641
Cae ~ bait	Negative binomial	3	1194.035
Cae ~ habitat+bait	Negative binomial	4	1177.565
Cae ~ habitat+(1 | site)	Negative binomial	4	1181.418
Cae ~ bait*habitat+(1 | site)	Negative binomial	6	1160.350
Cae ~ bait+habitat+(1 | site)	Negative binomial	5	1161.057
Cae ~ bait+(1 | site)	Negative binomial	4	1190.251

Letters in blue indicate the model selected for data analysis. 'Cae' represents the numbers of *Lucilia caesar* flies per trap per collection. '(1 | site)' indicates the inclusion of 'site' as a random factor in the given formula.

Table 5.8. Effects of 'habitat', 'bait' on the number of *Lucilia caesar* caught. The table displays the estimates, standard errors, z values an p values computed by the selected model.

Effects	Estimate	Std. Error	z value	p
Habitat(woods)	1.2247	0.2016	6.076	<0.001
Bait(toad)	-0.8410	0.1691	-4.972	<0.001

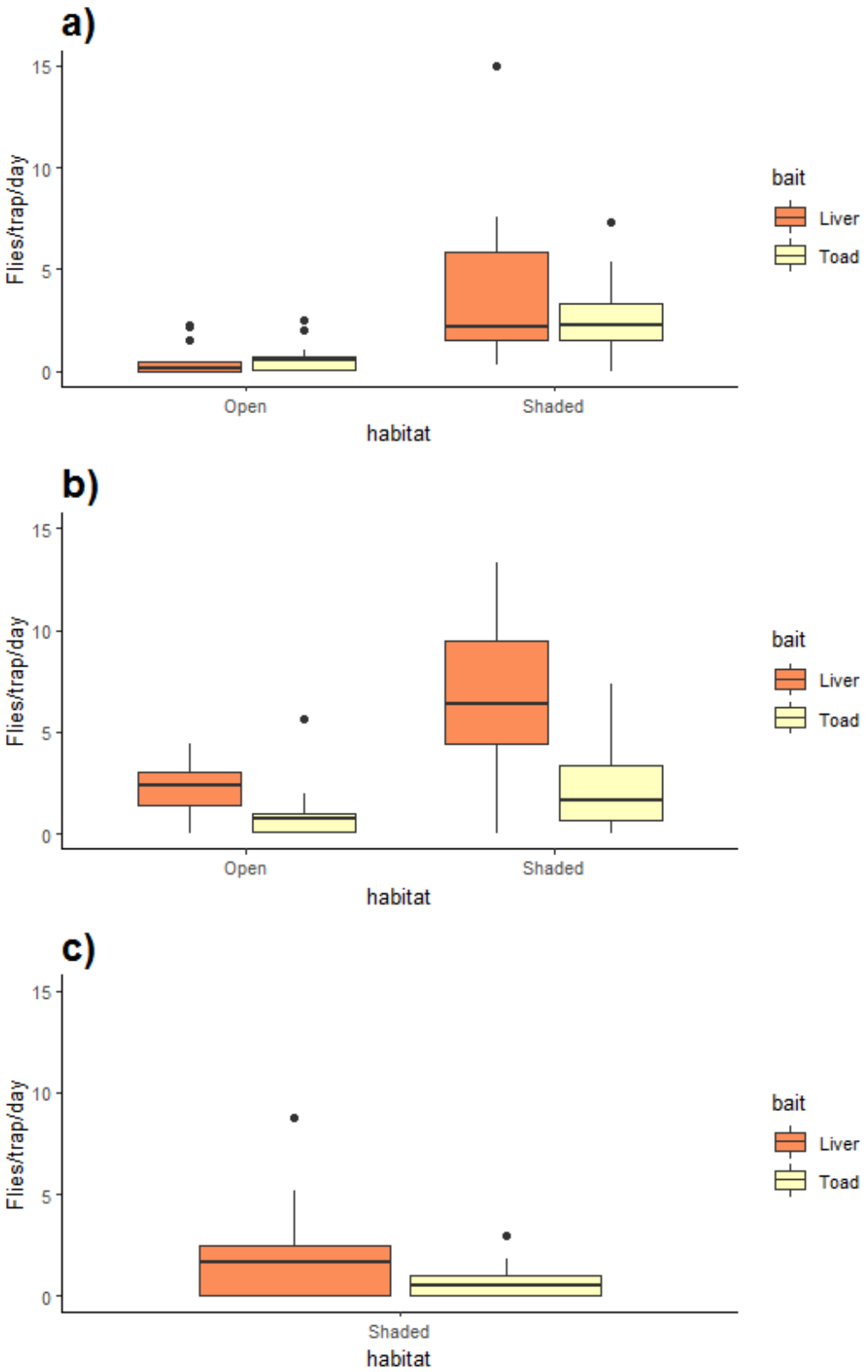

Figure 5.14. The number of *Lucilia caesar* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

Lucilia ampullacea

Model selected for *L. ampullacea* included fixed effects of 'habitat', 'bait' and random effects of 'site' (Table 5.9). The latter factor had a significant effect on fly-catch size ($\chi 2(1) = 38.42$, $P<0.001$). The lowest abundance recorded for this species was at site 1 (Fig. 5.15). This species was significantly more abundant in woodland habitats and its fly catch was significantly higher using liver-baited traps (Table 5.10, Fig. 5.15).

Table 5.9. AIC scores of the different models tested for data analysis of *Lucilia ampullacea* Table displays formula of the model, family, degrees of freedom and AIC scores.

Model	family	df	AIC
Amp ~ habitat	Negative binomial	3	764.283
Amp ~ bait	Negative binomial	3	769.186
Amp ~ habitat+bait	Negative binomial	4	745.945
Amp ~ habitat+(1 | site)	Negative binomial	4	733.907
Amp~bait*habitat+(1 | site)	Negative binomial	6	711.471
Amp~bait+habitat+(1 | site)	Negative binomial	5	709.527
Amp ~ bait+(1 | site)	Negative binomial	4	718.726

Letters in blue indicate the model selected for data analysis. 'Amp' represents the numbers of *Lucilia ampullacea* flies per trap per collection. '(1 | site)' indicates the inclusion of 'site' as a random factor in the given formula.

Table 5.10. Effects of 'habitat', 'bait' on the number of *Lucilia ampullacea* caught. The table displays the estimates, standard errors, z values an p values computed by the selected model.

Effects	Estimate	Std. Error	z value	p
Habitat(woods)	1.0041	0.2992	3.356	<0.001
Bait(toad)	-1.2230	0.2256	-5.420	<0.001

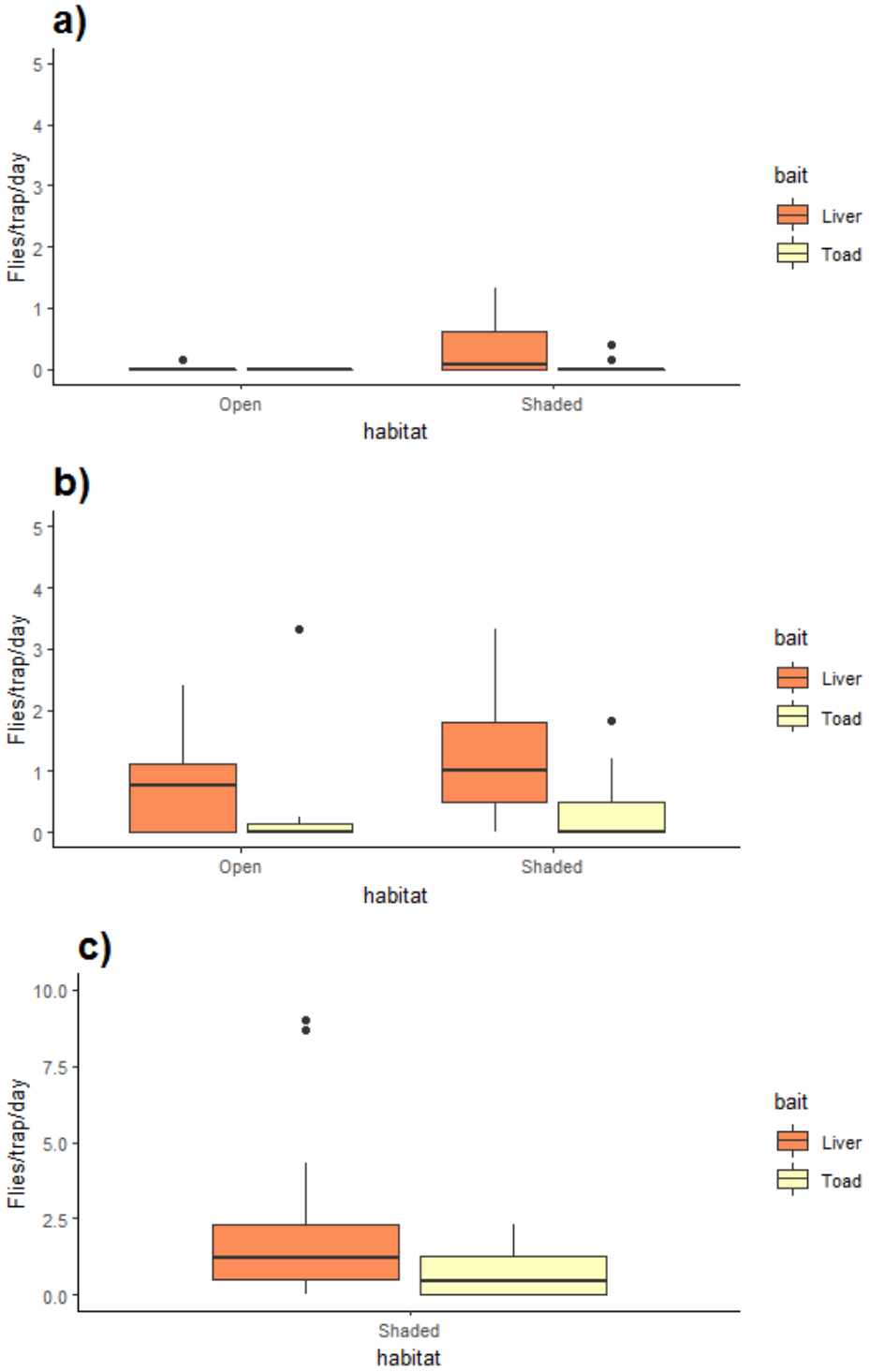

Figure 5.15. The number of *Lucilia ampullacea* caught (trap/day) in different habitats with different baits (Liver and toad) at different sites: a) site 1 – rural, b) site 2 – semirural, c) site 3 – urban. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

5.3.2 Effects of site

'Site' was a significant factor affecting the abundance of all species of *Lucilia* studied except for the sheep blowfly *L. sericata*, which was found at all sampling sites (Table 5.11, Fig. 5.16). In general, *L. caesar* was the most common species in the study, yielding a total of 1,726 flies caught (Table 5.11). On the other hand, the toadfly *L. bufonivora* was the least abundant species recorded. Nonetheless, it was more frequent at site 1 (Fig. 5.16). No specimens were caught at site 3 (Table 5.11, Fig. 5.16). Similarly, *L. silvarum* had its highest abundance at site 1, with a total of 117 flies caught and only two specimens caught at site 2 (Table 5.11, Fig. 5.16). *L. ampullacea* was a relatively rare species at site 1, however it was a common species at both site 2 and 3 (Table 5.11, Fig. 5.16). Finally, *L.illustris* was a common species at site 1 and 2, but with a very rare at site 3 site (Table 5.11, Fig. 5.16).

Table 5.11. Total numbers of flies caught at each study site. The total numbers of individual *Lucilia* species caught per site and the sum of all sites is displayed in the table.

Site	*L. bufonivora*	*L. silvarum*	*L. sericata*	*L. illustris*	*L. ampullacea*	*L. caesar*	Total
1.Olst	60	114	610	232	21	446	1483
2.Winssen	17	17	217	144	191	932	1518
3.Nijmegen	0	2	132	5	367	348	854
Total	77	133	959	381	579	1726	3855

5.3.3 Effects of 'habitat' and 'bait'

The fixed factor 'habitat' had a significant effect on the fly catch of all *Lucilia* species). The toad fly *L. bufonivora* was caught with more often in open areas (Fig. 5.17). This was also seen with its sister species, *L. silvarum* (Figure 5.17). *L. sericata* and *L. caesar* were the dominant species in open and shaded areas respectively (Figure 5.17). *L ampullacea* was more abundant in shaded areas (Figure 5.17). *L. illustris* was found in all habitats sampled but was significantly less abundant in forests (Fig. 5.17).

The factor 'bait' had significant effect on the fly catch of *L. bufonivora* or *L. silvarum*. Model selection removed this factor from analysis. *L. caesar*, *L. illustris* and *L. ampullacea* were caught more frequently by liver-baited traps than toad-baited traps (Figure 5.17). In woodland habitats, *L. sericata* was more abundant in liver-baited traps (Fig. 5.17).

5.4 Discussion

At the sites investigated in the Netherlands, adults of the amphibian parasite *L. bufonivora* were rare in comparison to other *Lucilia* blowflies such as *L. ceasar* or *L. sericata*, as would be anticipated from previous studies (MacLeod and Donnelly, 1956; Fischer, 2000). However, in the present study adult *L. bufonivora* were more abundant in non-shaded habitats (Fig. 5.10). This matches with the habitats on where toad myiasis cases are typically reported (Weddeling and Kordges, 2008; Gosá, et al., 2009). For instance, a study from Germany found numerous cases of amphibian myiasis from open habitats and rarely found cases in woodland habitats (Weddeling and Kordges, 2008). In the Iberian peninsula there are also reports of amphibian myiasis from open to semi-open landscapes of various natural parks (Gosá et al., 2009). Additionally, the specimens analysed by Tantawi and Whitworth (2014) were collected by net sweeping from flowers of *Achillea* and *Heracleum*. These plants are typically abundant in relatively open spaces such as abandoned grasslands, meadows, marshes, roadsides and forest edges (Page et al., 2006; Thiele et al., 2006; Alberski et al., 2009). Certainly, sampling site 3 (botanical gardens of Nijmegen) on which only shaded forests were surveyed, no specimens of *L. bufonivora* were found. These findings contrast with those from a study in Czech Republic that found *L. bufonivora* typically more abundant in forests (Fischer, 2000).

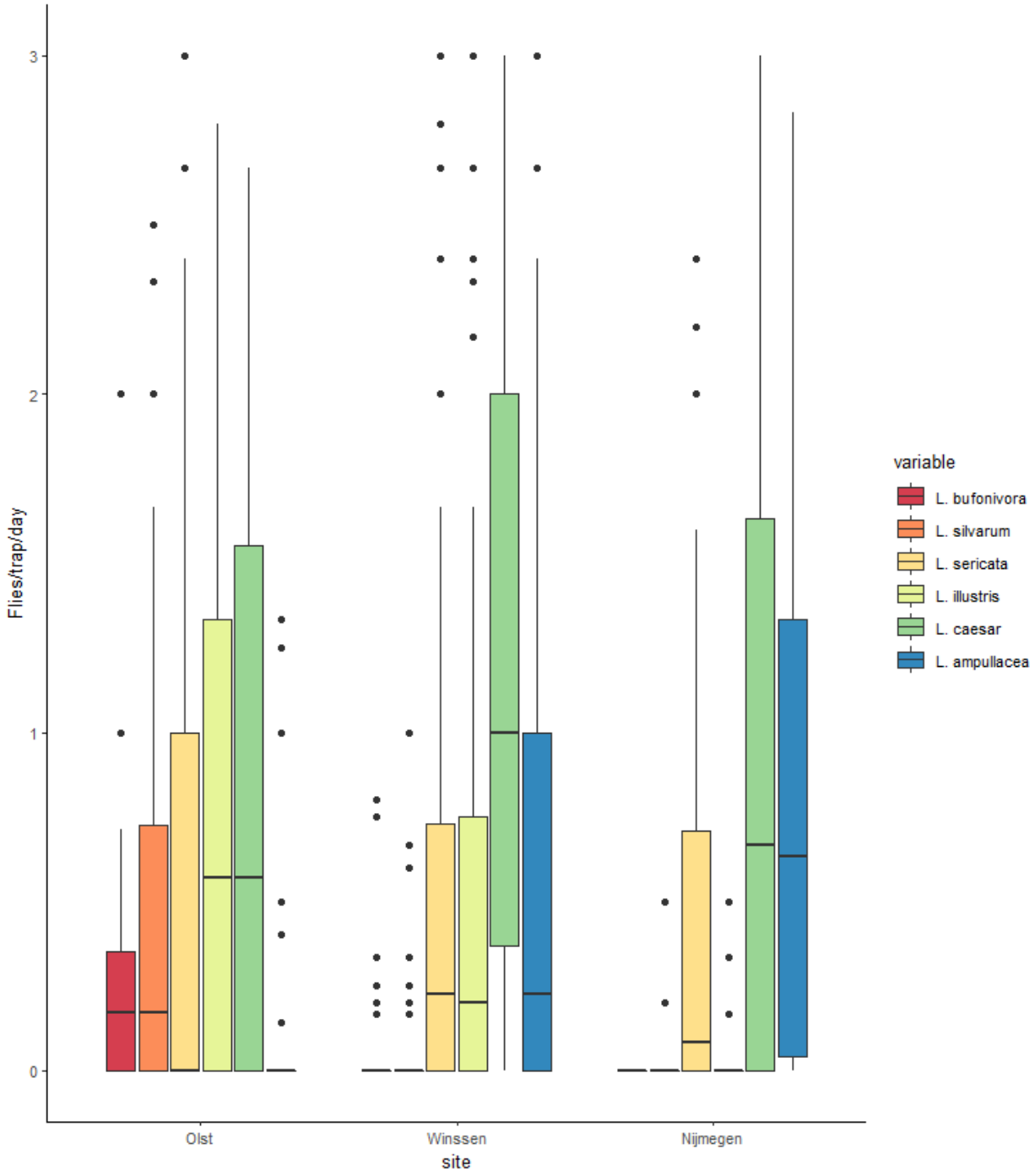

Figure 5.16. The number of various species of *Lucilia* caught (trap/day) at different sites: site 1 (Olst), site 2(Winssen) and site 3 (Nijmegen). The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

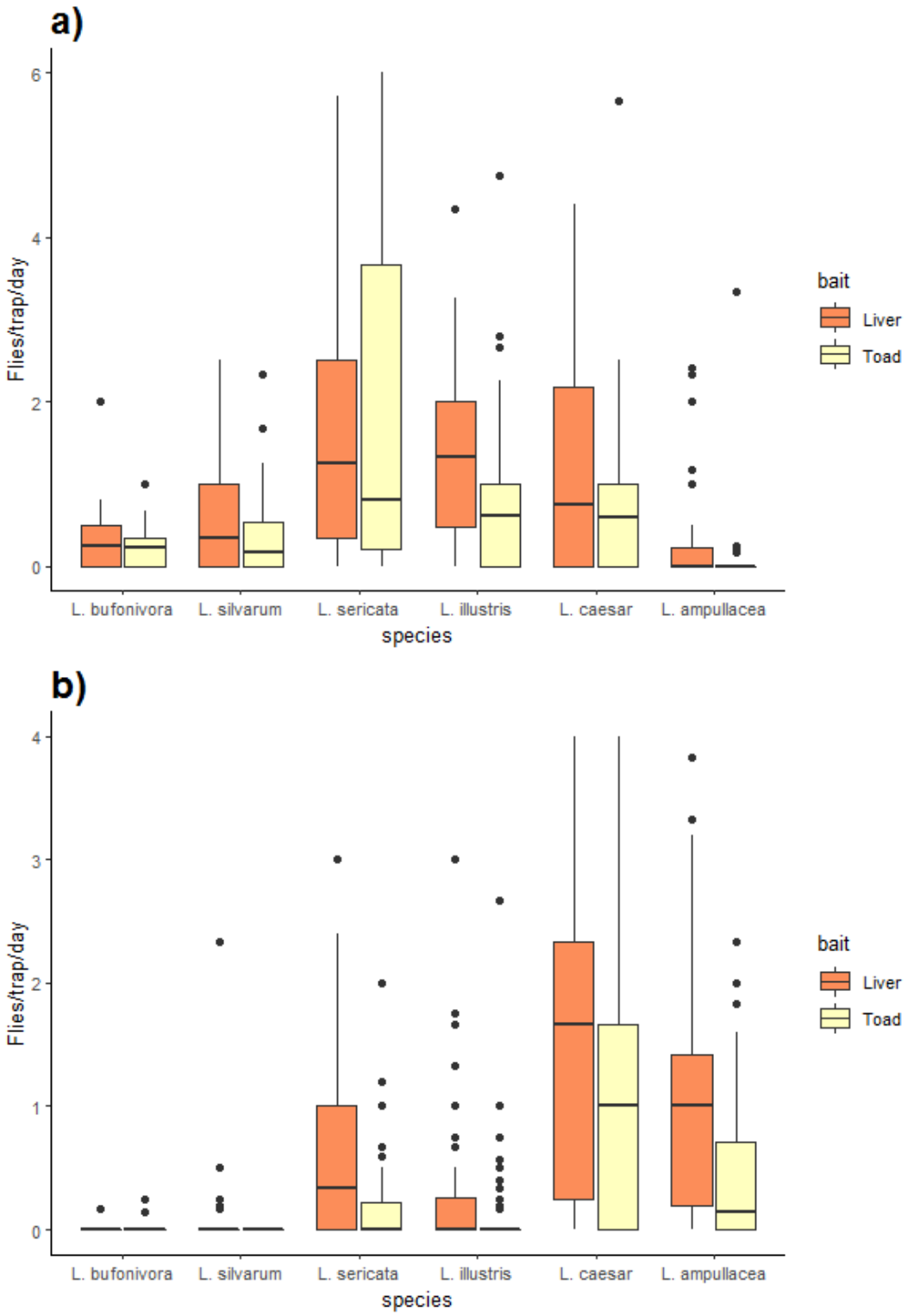

Figure 5.17. The number of various species of *Lucilia* caught (trap/day) with different baits (liver and toad) at different habitats: a) open and b) shaded. The median flies/trap/day is displayed within boxes representing first and third quartiles. Whiskers show 95% confidence intervals with outliers (dots).

To a large extent the presence of *L. bufonivora* is likely to be dictated by the presence of open water and its amphibian hosts, rather than its own immediate habitat requirements. This study and previous reports of toad myiasis suggest that the activity of *L. bufonivora* is higher at open or semi-open landscapes (Fig. 5.10). Although *L. silvarum* has different larval feeding habits from its sister species *L. bufonivora* (Fremdt and Amendt, 2014), this study showed that they are both more frequent in the same type of habitats (Fig. 5.11). This matches with previous research that found *L. silvarum* to be more abundant at open or semi-open sunny habitats with meadow and grassland vegetation (MacLeod and Donnelly, 1956; Aesch et al., 2003). Therefore, in addition to the close relationship morphologically and phylogenetically with *L. bufonivora* (Rognes, 1991; McDonagh and Stevens, 2011), these species have also very similar adult-fly behaviour.

It is known that the metabolic processes and larval development of blowflies are temperature dependant (Wall et al., 1992). Hence, body temperature in mammals provides optimal conditions for their development, for example in sheep skin surface temperature has been estimated at 37 °C (Davies and Hobson, 1935). Certainly, the host range of parasitic blowflies are mostly mammals (Zumpt, 1965; Stevens and Wallman, 2006; Stevens et al., 2006). However, amphibians are ectothermic vertebrates and cannot regulate their temperature metabolically, hence field temperatures strongly influence their physiology and survivorship (Brattstrom, 1979; Hutchison and Dupre, 1992) although body temperature of toads is highly correlated with the extent of exposure to solar radiation and basking behaviour (Careay, 1978; Meek and Jolley, 2006). In fact, a study on *B. bufo* found body temperatures of above 30°C in basking individuals (Meek and Jolley, 2006). Although there is very little known about the temperature requirements of the toad fly, toads with warmer body temperatures that are basking in sunny areas could be more likely to serve as a host to *L. bufonivora*. This, however, needs to be confirmed with further research on the physiological processes and host-selection of this species.

Field work from chapter 2 revealed that *L. caesar* is the most abundant calliphorid species in South West England. Similarly, present results indicate that this species is also the most abundant calliphorid from the sites investigated in the Netherlands and the data from both countries showed that it is the dominant species in shaded habitats. This has been well recorded in the past (MacLeod and Donnelly, 1956; Gregor, 1991; Smith and Wall, 1997b). Similarly, *L. sericata* was the most abundant *Lucilia* species in non-shaded habitats, which also matches with results from Chapter 2. This suggests that *L. bufonivora* and *L. silvarum* co-exist

with potential stronger competitors of the carrion fly community (Hanski and Kuusela, 1977), which could have played an important role on the sympatric speciation of *L. bufonivora*.

The type of carrion can influence oviposition and colonisation (Lane, 1975; Smith, 1986; Smith and Wall, 1997b; Heath and Appleton, 1999; Byrd and Castner, 2001). However, the results presented here did not show any association with bait type; adult *L. bufonivora* were equally abundant in both liver and toad-baited traps. This contrasts with the speculation by Zumpt (1965) which suggested that adult females of this species are attracted to feeding on dead toads. To start vitellogenesis, newly emerged blowflies need a proteinaceous meal which can be usually obtained from carrion (Wall, 1992; Huntington and Higley, 2010). Hence, *L. bufonivora* might use any available carrion as a protein source for vitellogenesis, which in this case could have been obtained from both baits used in this study. However, no gravid females of *L. bufonivora* were observed in the traps and, due to its life-history trait as an obligate parasite, it would not use this source for oviposition (Brumpt, 1934). This might explain the low abundance of *L. bufonivora* using carrion as a bait, as it lays eggs exclusively on a live host (Weddeling and Kordges, 2008). In contrast, the present work found adult flies of saprophagous blowflies (e.g. *L. illustris, L. caesar* and *L. ampullacea*) more abundant in liver-baited traps. Similarly, when present in woodland, *L. sericata* was more abundant in traps that were baited with liver. Typically, saprophagous female blowflies that are attracted to carrion-baited traps are either gravid flies ready for oviposition or young flies in search of a proteinaceous meal (Brodie et al., 2014). Certainly, gravid and non-gravid females are attracted to semiochemicals from flies that are either ovipositing (gravid) or feeding (non-gravid) on the same resource (Brodie et al., 2015). Hence, the cue response by gravid and non-gravid females of *L. bufonivora* might be very different to the one exhibited by saprophagous blowflies. Previous research on *L. sericata*, has shown that, not only chemical, but also visual cues are important when selecting a final landing site (Wall and Fisher, 2001). Adult oestrid flies of *Cephenemyia trompe* and *Hypoderma tarandi*, obligate parasites of reindeer, are more attracted to mobile targets than to stationary ones (Andersson and Nissen, 1996). Host-seeking behaviour of frog-biting midges, *Corethrella* spp., strongly depends on acoustic cues from their anuran hosts (Bernal and de Silva, 2015). To date, the cues that are involved in the attraction of gravid females of *L. bufonivora* are unknown and more research is required to resolve the host-seeking behaviour of this obligate parasite.

In general, the present results on habitat use by *Lucilia* blowflies match those observed in Chapter 2. However, species composition varied between countries. For instance, this work did not recover a specimen of *L. richardsi* and, although low in abundance, Chapter 2

confirmed its presence in the South-West UK. Similarly, this work found *L. illustris* a relatively common species from the Netherlands, however it was very rare in UK (Chapter 2). While no specimens of *L. bufonivora* were collected in the UK, this study recovered 77 specimens from two different sites of the Netherlands.

In conclusion, using carrion-baited traps, adult *L. bufonivora* is a rare species compared to other species such as *L. sericata* or *L. caesar*. However, this low abundance might be the product of its specialized behaviour as an obligate parasite, using carrion as a vitellogenic protein source rather than a breeding site. This species was significantly more abundant at open and semi-open sunnier areas and very low abundant in shaded habitats such as woodlands or forests. It was equally attracted to toad carcasses than it was to standard blowfly baits, such as liver. Although this work provides a better understanding on its spatial distribution, its temporal abundance remains unsolved. More research that uses a longer trapping period (e.g. early spring to late summer) is required to understand this. It could provide valuable data on the number of generations per year, temperature effects on its abundance and also to understand whether its phenology and life-cycle is related with that of its hosts.

6. General Discussion

6.1 Habitat partitioning by blowflies

Calliphorid blowflies are one of the best known, commonly encountered and economically important groups of insects due to the ecosystem services they provide as consumers of carrion. Ephemeral resources, such as a carcass, facilitates intense interspecific competition among the individuals that use it for development (Hanski and Kuusela, 1977; Hanski, 1987). Thus, the coexistence of blowflies is driven by niche differentiation mediated through differences in phenology, synanthropy, type of carrion and environmental tolerance to factors such as humidity and light intensity (Hanski and Kuusela, 1977; Smith and Wall, 1997b; Cruickshank and Wall, 2002; Hwang and Turner, 2006).

This study found *Calliphora* species to be more abundant in cooler months, which reflects their low temperature requirements (Greenberg, 1991). Segregation between *Calliphora* and *Lucilia* species appears, therefore, to be mediated by season and temperature. In UK ecosystems, *Calliphora* species are the first blowflies to emerge in spring, which has also been found in previous research on the seasonal variation of calliphorid flies (Greco et al., 2014; Zabala et al., 2014). Clear knowledge of the seasonal distribution of blowflies in different geographical ranges is not only of ecological relevant, but it also provides valuable data which could later be used in supporting evidence for legal cases and thus, is of importance to forensic sciences.

Out of seven species of *Lucilia* reported in the UK (Emden, 1954; MacLeod and Donnelly, 1956), only six were found in this study (*Lucilia sericata*, *Lucilia illustris*, *Lucilia richardsi*, *Lucilia caesar*, *Lucilia silvarum* and *Lucilia ampullacea*). *Lucilia bufonivora* was the only species absent in the South West UK. Its presence has been confirmed, however, in other localities of UK (e.g. Norfolk, Suffolk, etc.) using DNA-based identification methods (McDonagh and Stevens, 2011). Interspecific segregation of the different species of *Lucilia* appears to be mediated by factors that are defined by the type of habitat, such as light intensity and humidity levels. Certainly, habitat had the strongest influence over the abundance and distribution of the different species of *Lucilia*. For instance, *L. caesar* was the dominant species in shaded areas like woodland and microhabitats that provide shaded environments such as hedgerows but certainly it was less frequently found in open pasture. This species segregation has been well reported in the past (MacLeod and Donnelly, 1956; Smith and Wall, 1997b; Martínez-Sánchez, et al., 2001). Overall, *L. caesar* was the most abundant calliphorid fly found at different sites in South West UK and the Netherlands.

On the other hand, open pasture habitats were dominated by the sheep blowfly *L. sericata*. This indicates that it is confined to locations with lower humidity levels and high light intensities. Thus, as previously discussed, niche differences might have played an important role on the evolution of ectoparasitism of *L. sericata*, with this species being able to colonize live hosts in open areas. Though it is the most common species involved in ovine cutaneous myiasis in the UK (Wall et al., 1992a), it is still unclear why this species typically exhibits saprophagic behaviour outside of this range. This phenomenon, however, might be related to niche availability in different geographical locations. For instance, in Mediterranean countries the flesh fly *Wohlfahrtia magnifica* (Sarcophagidae) develops as an obligate agent of cutaneous myiasis, where it may effectively exclude *L. sericata*. Similarly in Australia, the myiasis niche seems to be occupied by *Lucilia cuprina*, which is also able to effectively exclude *L. sericata*. More detailed studies are needed to understand the intraspecific behavioural variation in different populations of the sheep blowfly *L. sericata*.

A sympatric species, *L. richardsi* largely resembles *L. sericata* in morphology (Rognes, 1991), and was found to be most abundant in the same type of habitats as *L. sericata* - mostly open landscapes. Although *L. richardsi* is not involved in ovine myiasis, Nuorteva (1959) reported it from myiasis wound of a nightjar. Since then, there are no existing records of this species parasitizing animals. In fact, in some instances, *L. richardsi* may be a species of forensic importance, as shown by a study that described the morphological features of its larval stages that were obtained from females laying eggs in carrion (Szpila et al., 2013). In contrast with *L. sericata,* this study found that *L. richardsi* is rarely encountered in the field. Despite their known close morphological, genetic and behavioural similarity with *L. sericata* it is still unclear why the latter species has never been recorded causing sheep myiasis; as with the examples given above, competitive exclusion could be one possible reason – but this would require experimental study to resolve.

Although this work could not evaluate the blowfly seasonal variability in the Netherlands, the blowfly species diversity from this country was relatively different to the one observed in South West UK. For instance, *L. illustris,* a closely related species to *L. caesar,* was a relatively common species in the Netherlands, however very rarely encountered in England (with no more than 10 specimens caught for the whole trapping season). In fact, no statistical analysis could be carried out with the data obtained from UK because of the low numbers of this species. Nonetheless, data from the Netherlands demonstrated that it was less common in shaded areas than its sister species *L. caesar*. Although it does not appear to have

close relationships with *L. sericata*, *L illustris* is occasionally involved in sheep myiasis in Europe (Brinkmann, 1976) .

The saprophagous species *L. silvarum* was a very rare species in the UK. The field work carried out in the Netherlands, however, showed that it was relatively abundant in the month of August. This species was almost never caught at woodland habitats, which was a feature shared with its sister species the obligate agent of myiasis *L. bufonivora*. The absence of the latter species in the surveyed areas from UK also might reflect the low abundance of potential hosts (e.g. *Bufo bufo*). This suggests that, as might be expected, *L. bufonivora* is confined to areas where its most common host *B. bufo* is also abundant, a point also noted by Fischer (2000). Certainly, *L. bufonivora* was caught in sites from the Netherlands where relatively large populations of *B. bufo* were available. Contrasting with the findings of Fischer (2000), however, the current work found that *L. bufonivora* was more abundant in open spaces, rather than forests and woodland.

6.2 Why misidentification of *Lucilia* blowflies is so common?

Lucilia is a relatively small genus of blowflies that morphologically resemble each other closely (Rognes, 1991). Misidentification is a very common phenomenon and it often leads to erroneous reports in biodiversity data (Rognes, 2014). Part of this is due to the taxonomic confusion of the monophyly of *Lucilia,* which has been debated for decades (Rognes, 1991; Stevens and Wall, 1996; Williams et al., 2016). Typically, one of the main factors that has exacerbated this issue has been the use of synonymic genera in different geographical regions, particularly North America, with the genera *Bufolucilia*, *Phaenicia*, *Hemipyrellia,* proposed[1]. Recent phylogenetic analyses have shown that *Hemypyrellia*, for instance, should be dismissed as a synonym to *Lucilia* (Williams, et al., 2016). The present work also supports the monophyly of *Lucilia*, as it was found to be paraphyletic with respect to another genus proposed by Townsend (1919) *'Bufolucilia'*. The latter genus, therefore, should also be dismissed as a synonym to *Lucilia*. Although the monophyly of *Lucilia* seems to become stronger with the improvement of phylogenetic methods, more analyses with samples from across the globe are still required to fully resolve the monophyly of *Lucilia*.

[1] See Townsend (1919) and Hall (1948)

In addition, morphological differences displayed by recently diverged taxa are sometimes minimal. For instance, identification of the sheep blowflies *L. sericata* and *L. cuprina* using morphological features extremely difficult. Moreover, it is known that these species undergo hybridisation (Stevens and Wall, 1996b; Williams and Villet, 2013). In some other species even identification with molecular markers can be difficult, as reported for the sister taxa *L. illustris* and *L. caesar* (Sonet et al., 2012). Nonetheless multi-gene approaches seem to overcome this problem. For instance, in combination with *COX1* sequence data, the BI *per* gene phylogeny from the present study could contribute solving the species delimitation issue for the latter two species. The phylogenetic relationships of *L. caesar* and *L. illustris,* however, needs further exploration

One of the major factors, clearly identified from this study, is the ongoing taxonomic confusion of *L. bufonivora* with *L. silvarum*. Morphological identification can be extremely difficult. Specially because one of the features for species level identification given in morphological keys (Emden, 1954) is the number of *post acr* bristles (2 in *L. bufonivora* and 3 in *L. silvarum*). This, however, is highly variable between individuals (Rognes, 1981), and might have contributed largely to the continuous misidentification of these taxa. Species identification, therefore, cannot rely on this morphological feature and instead it should be determined by examining genitalia as indicated by Rognes (1991). Clearly, to avoid further confusion, accurate identification should be performed, not only by detailed morphological examination, but also confirming identity using a DNA-based multi-gene approaches.

6.3 What is the taxonomic status of the toad fly *L. bufonivora?*

All BI phylogenies inferred in this thesis support the status of *L. bufonivora* as a distinct species to *L. silvarum* and *L. elongata*. In arthropods, species delimitation is typically attributed to monophyly but also to phenotypical and genotypical features that enable unambiguous differentiation and diagnosis (Dantas-Torres, 2018). Certainly, identification of *L. bufonivora* can be performed unambiguously using both molecular data (e.g. *COX1* and *ITS2*) and with morphological features (Rognes, 1991). A previous phylogenetic study also grouped *L. bufonivora* and *L. silvarum* as distinct species using mtDNA (McDonagh and Stevens, 2011). The same study, however, failed to differentiate them using nuclear DNA (*EF1a* and *28s*). Similarly, this present study also found no clear resolution on the relationships of *L.*

bufonivora, *L. silvarum* and *L. elongata* when using the *EF1-EF4* region of the nuclear gene *EF1a*. Thus, *EF1a* does not appear to be a suitable marker for inferring relationships of recently diverged taxa. Nevertheless, newly optimized nuclear molecular markers that have shown to be useful for inferring relationships at species (Marinho et al., 2011; Williams and Villet, 2013), provided enough evidence to solve this taxonomic issue. Indeed, all taxa appear to exhibit a unique haplotype of the gene *ITS2*. Similarly, the protein coding gene *per* provided higher phylogenetic resolution than *EF1a*. In combination, *ITS2* and *per* provided nDNA evidence to infer accurately the close relationships of *L. bufonivora*, *L. silvarum* and *L. elongata*, thus, grouping them as distinct sister species. These conclusions, however, are based on only two nuclear loci and one mitochondrial locus. Given the recent advances of phylogenomics in dipterology (Kutty et al., 2019), more detailed studies using these tools are required for deeper insights on the relationships of these closely related taxa.

6.4 Which species is/are involved in amphibian myiasis?

Results from this work suggest that, in Europe, amphibian myiasis seems to be caused exclusively by *L. bufonivora*. Although *L. silvarum* was thought to be involved in this condition its saprophagic behaviour has been well recorded in the past (Hanski and Kuusela, 1977; Hanski, 1987; Prinkkila and Hanski, 1995; Fremdt, et al., 2012). This work suggests, as previously argued by Zumpt (1965), that reports of *L. silvarum* causing amphibian myiasis in Europe are likely to be the result of misidentification with *L. bufonivora*. For instance, using DNA-based identification methods, the present work showed that all European sequences from larvae that had been found infesting live amphibians belonged to *L. bufonivora*. Indicating, therefore, that *L. silvarum* is not involved in amphibian myiasis in Europe. It is known that amphibians that host larvae of *L. bufonivora* do not usually survive infestation (Brumpt, 1934; Strijbosch, 1980). Accurate diagnosis of the species composition in amphibian myiasis should, therefore, be performed by analysing the larvae that are found causing the disease in the live host. Given that blowflies are the first group of insects to colonize carrion (Hall, 2001), amphibian carcasses might also serve as a food source to other saprophagous blowflies. A study from Germany recovered 53 cases of amphibian myiasis which, after death, were left under natural conditions to decompose (Weddeling and

Kordges, 2008). The authors found a wide variety of saprophagous flies emerging from the carcasses (e.g. *C. vicina, L. sericata*) as well as specimens of toad fly *L. bufonivora*. The mentioned study, however, found no specimens of *L. silvarum* emerging from such carcasses.

In North America, however, *L. silvarum* has been reported as causing amphibian myiasis (Roberts, 1998; Bolek and Coggins, 2002; Bolek and Janovy, 2004; Eaton, et al., 2008). These reports remain questionable, as the results may not be reliable, given that none of these studies have used molecular methods; and again, taxonomic confusion and misidentification might be the reason to this. For instance, although *L. bufonivora* was thought to be absent in the Nearctic, Zumpt (1965) stated "*L. bufonivora* may occur in North America, where it is perhaps confused with *L. silvarum*, but this is a problem that remains to be cleared up.". Indeed, *L. bufonivora* remained unrecorded in this area until Tantawi and Whitworth (2014) confirmed its presence in Canada and noted that it has been confused with *L. silvarum* since 1954. To illustrate this problem, two samples reared from different amphibian myiasis cases in Canada were provided to the author, labelled as '*L. silvarum*'. These samples have been identified using North American keys which do not include *L. bufonivora* (Hall, 1948). Subsequent DNA analysis revealed their identity as *L. bufonivora* and, thus, Hall's keys can potentially lead to the misidentification of the latter species – and should not therefore be used. Unfortunately, in North America, these keys are commonly used for identification of flies that are found involved in myiasis. Results from the present thesis, therefore, suggest that records of *L. silvarum* involved in amphibian myiasis in North America might also due to misidentification of *L. bufonivora*. Further studies that employ molecular and morphological methods are required to confirm the species composition in amphibian myiasis in North America. Nevertheless, this present work is the first study to confirm the involvement of *L. bufonivora* in amphibian myiasis in Canada using DNA-based identification methods. The species found affected were the wood frog, *Lithobates sylvaticus* and the western chorus frog, *Pseudacris triseriata*.

6.5 Evolution of ectoparasitism in *Lucilia* and origins of obligate amphibian parasitism

Previous research suggests that blowflies in general have evolved ectoparasitism independently several times (Stevens and Wall, 1997; Stevens, 2003; Stevens and Wallman, 2006; McDonagh and Stevens, 2011). Moreover, the evidence suggests that the independent

evolution of ectoparasitisim appears even within taxa that comprise the genus *Lucilia*. Facultative myiasis agents of livestock (e.g. *L. sericata* and *L. cuprina*) are thought to have evolved parasitism in association with man and sheep domestication (Erzinclioglu, 1989; Stevens and Wall, 1997). The reason to this is that myiasis is rarely reported affecting hosts in the wild. Both species are estimated to be at least 4 mya old, which is also supported by the present time-scaled phylogeny. This suggests, therefore, that *L. sericata* and *L. cuprina*, had predominantly saprophagous feeding habits, perhaps occasionally infesting dying or debilitated live animals, before the domestication of sheep, which has been estimated to happen 11 000 years ago (Zeder, 2008). As previously discussed, sheep husbandry could have provided a suitable and unoccupied alternative niche for *L. sericata* and *L. cuprina* in different geographical ranges. Obligate amphibian parasitism, however, differs largely to sheep facultative myiasis. Firstly, it affects wild hosts to a greater extent. Secondly, it has a higher pathogenicity, host-specificity and most of the times it causes the death of the host (Brumpt, 1934; Strijbosch, 1980). Finally, the physiological processes of amphibians are very different to those of the mammals; body temperature for instance. More studies on the physiology of the toad fly are required to understand the traits that have enable this species' niche adaptation.

In contrast with the independent evolution of facultative ectoparasitism across the genus *Lucilia,* obligate amphibian parasitism appears to have evolved only once. It was recovered as a monophyletic life history trait in mtDNA (*COX1*) and nDNA (*ITS2* and *per*) phylogenies and supported by the time-scaled phylogeny. Within *Lucilia* this behaviour is only displayed by *L. bufonivora* and *L. elongata*, however their close relationship with the saprophagous *L. silvarum* suggest that it diverged from a generalist saprophagous ancestor. It is likely that, in combination with interspecific physiological requirements, the intense competition within the carrion-fly community mediated the migration of *L. bufonivora* to this narrow and unoccupied niche. Certainly, the present-time scaled phylogeny suggests that this evolutionary event occurred approximately 5mya by showing a clear split between a saprophagous lineage (*L. silvarum*) and a strictly obligate lineage (*L. bufonivora* + *L. elongata*). This is relatively recent compared with the evolution of other major Diptera groups that exhibit strictly obligate habits such as Oestrid flies (Stevens et al., 2006). In fact the diversification of Oestridae is thought to be associated with the major radiation of mammals during the Paleogene (30-50mya) (Pape, 2006; Junqueira et al., 2016). Given that all taxa that comprise Oestridae display obligate parasitism, coevolution and host-parasite interactions might have played an important role in their speciation (Stevens and Wallman, 2006).

Nevertheless, as discussed before, evolution of obligate amphibian parasitism was a result of niche displacement rather than host-parasite coevolution.

The reciprocal monophyly between Nearctic and Palearctic parasites of amphibians suggest that their most recent ancestor already exhibited obligate parasitic habits. This life history trait, therefore, evolved before the intercontinental dispersion of this ancestral state rather than the independent origin of obligate amphibian parasitisim in two different continents. It is unclear, however, how this intercontinental dispersal occurred and more phylogeographic studies are required to answer this question. The monophyletic origin of obligate amphibian parasitism in *Lucilia* is in contrast with other blowflies that seem to have evolved obligate parasitism of mammals independently. The most representative example is the new-world and old-world screwworm flies, *Cochliomyia hominivorax* and *Chrysomya bezziana* respectively (McDonagh and Stevens, 2011).

Subsequent geographical isolation triggered then the speciation of *L. bufonivora* in the Palearctic and *L. elongata* in the Nearctic. Although this work confirmed the presence of *L. bufonivora* in Canada, the time scaled phylogeny indicates that this cannot be attributed to a recent introduction to this range. Certainly, the split between *L. bufonivora* and *L. elongata,* which occurred in the Nearctic, was estimated to happen approximately 2mya. *Lucilia bufonivora* then remained rather unrecorded due to its low abundance and the already intensely discussed taxonomic confusion of this species group.

6.6 On-going cryptic speciation of *L. bufonivora*?

As stated before, *L. bufonivora* is confirmed to be present in the North American continent. High rates of mtDNA sequence divergence and consistent paraphyly across nDNA phylogenies suggest cryptic speciation of *L. bufonivora* in the Nearctic. Surprisingly, toad fly samples from Canada exhibited a closer affinity to the strictly Nearctic *L. elongata*.

Lucilia bufonivora (Europe), *L. bufonivora* (Canada) and *L. elongata* exhibited unique haplotypes of the non-coding gene *ITS2* with differences in length and base composition. In combination with sequence data from the mtDNA marker *COX1,* unambiguous identification of these three putative taxa can be performed accurately. Current findings suggest, therefore, the presence of a cryptic species of *L. bufonivora* in North America. Nevertheless, thorough morphological examination with a wider sample size is required to

determine whether *L. bufonivora* is indeed a different species in the Nearctic or simply it corresponds of a species complex comprised by a Palearctic and a Nearctic subspecies.

The *COX1* phylogenies presented in this work suggest that, indeed, arthropod mtDNA displays faster evolution rates than nuclear DNA. Certainly, the lack of recombination and accumulation of mutations enable the fast evolution of mtDNA (Brown et al., 1979; Neiman and Taylor, 2009; McDonagh et al., 2016). Within the *L. bufonivora* species group, however, the mtDNA evolution rate appears to be even faster compared with other species of *Lucilia*. For instance, the intraspecific genetic distance observed between Canadian and European *L. bufonivora* was rather high. In fact, it was greater than the interspecific distance shown by *L. sericata* and *L. cuprina*. As discussed in the previous chapter, the attributes of *L. sericata* such as high fertility, migration capacity, synanthropy and facultative myiasis behaviour, reduce potential gene flow barriers, thus, reducing the impact of genetic drift and finding strong genetic consistency across geographically distant samples. In contrast, species that exhibit a highly specialised behaviour and have a restricted dispersal capacity are more vulnerable to genetic drift. This work provides enough evidence to suggest the independent evolution of two geographical isolated populations of *L. bufonivora*. A similar case has been reported for a fleshfly that causes obligate myiasis in a range of non-livestock hosts, *Wohlfartia vigil*, that appears to have at least two different species (or sub-species) according to its geographical range (Hall et al., 2009). Given, that it is unlikely to be dispersed by human activity, genetic isolation is more likely to occur. There are still, however, many aspects of this issue that need further exploration, such as mitochondrial heteroplasmy and even cytoplasmic incompatibility induced by *Wolbachia*.

In conclusion, given the wide variety of life histories of Calliphoridae, phylogenetic and ecological studies are of great importance for veterinary, evolutionary, medical and forensic sciences. Misidentification and taxonomic confusion, however, have been limiting factors for the studies of these species and the evolution of myiasis and for research in dipterology in general. This thesis highlights the use of recent molecular techniques and methods that are gradually helping to overcome this problem; such as multi-gene approaches for identification of blowflies or choosing the right statistical models to predict accurately their distribution and abundance. Using negative binomial distribution modelling, results suggest that the coexistence of blowflies is indeed mediated by differences in their phenology, temperature and humidity requirements, thus resulting in effective habitat partitioning. Carrion, as an ephemeral resource, creates intense interspecific competition that has forced certain species to migrate, in an evolutionary sense, to different niches. It can be argued that

it is the patchy and ephemeral nature of carrion that is perhaps the key to understanding the ecology and evolution of this family of flies. Human activity and sheep domestication offered an unoccupied niche that potentially triggered the evolution of facultative ectoparasitism of several taxa independently, as displayed by the primary agents *L. cuprina* - *L. sericata* and the secondary agents *L. caesar* and *L. illustris*. Some other species, however, were able to evolve parasitic behaviour independently from animal domestication and likely as a result of the niche displacement of the carrion-fly community, such as the toad fly *Lucilia bufonivora*. This work also highlights the role of geographical and ecological isolation in speciation within taxa that exhibit highly specialised behaviour, such as obligate amphibian parasitism. There are still many issues to be researched, such as the behavioural differences associated with the distribution *L. sericata*; and a broader study of the monophyly of *Lucilia* itself would be of value.

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Appendix I: Publications

- **Arias-Robledo, G.,** Stark, T., Wall, R., Stevens, J. (2019) The toad fly *Lucilia bufonivora*: its evolutionary status and molecular identification. *Medical and Veterinary Entomology*, **33**:131-139 https://doi.org/10.1111/mve.12328

- **Arias-Robledo, G.,** Stevens, J., Wall, R. (2019) Spatial and temporal habitat partitioning by calliphorid blowflies. *Medical and Veterinary Entomology*, **33**:228-237 10.1111/mve.12354

- **Arias-Robledo, G.,** Wall, R., Szpila, K., Shpeley, D., Whitworth, T., Stark, T., King, R.A., Stevens, J.R. (2019) Ecological and geographical speciation in *Lucilia bufonivora*: Evolution of amphibian obligate parasitism. *International Journal for Parasitology: Parasites and Wildlife.* https://doi.org/10.1016/j.ijppaw.2019.09.005